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# IDENTIFICATION OF CHEMICAL OVIPOSITION STIMULANTS FROM RICE GRAIN FOR *Sitophilus zeamais* MOTSCHULSKY (COLEOPTERA, CURCULIONIDAE)

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**Abstract**—Chemical components stimulating oviposition by *Sitophilus zeamais* in rice grain were isolated from rice bran and were found to be a mixture of ferulates, diglycerides, and free sterols. Oviposition preference of the species can be induced by synergistic action of these compounds.

**Key Words**—Rice weevil, *Sitophilus zeamis* Motschulsky, oviposition preference, ferulates (24-methylenecycloartanyl, cycloartenyl, sitosteryl, campesteryl), diglycerides (1,2-dilinoleic, 1,2-palmitic and linoleic), and sterols (campesterol, sitosterol, stigmasterol).

## INTRODUCTION

*Sitophilus zeamis* (Curculionidae), a most destructive pest of rice, corn, other grains, and their processed products is worldwide in distribution from tropical areas to temperate zones. To clarify the ovipositional behavior of the insect, we decided to investigate chemical oviposition-stimulating substances from rice brans. Honda et al. (1969) reported the food attractants from rice and corn for the rice weevils as hexanoic and heptanoic acids, nonan-4-olide, 2-nonen-4-olide, and 2-phenylethyl alcohol. The food attractants, however, have no oviposition activity for rice weevils. Recently, Arakaki and Takahashi (1982) reported the oviposition preference of rice weevils for polished,



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unpolished, parboiled polished, and parboiled unpolished rice and suggested the presence of the oviposition-stimulating substances in the methanol extracts of the rice bran. We have investigated the structure of the oviposition stimulants for the rice weevils.

## METHODS AND MATERIALS

*Insects*. The strain of rice weevil used in this study was kindly supplied by Professor T. Yoshida of the College of Agriculture, Okayama University, Japan. The rice weevils were reared in glass bottles (5 cm in diameter, 10 cm high, and 225 ml volume) covered with a 40-mesh wire net (3 cm in diameter) for aeration. The conditions of rearing were as follows: temperature 30° C, atmospheric moisture 72% relative humidity, illumination 18 hr light, 6 hr dark.

*Thin-Layer Chromatography (TLC)*. TLC was carried out on 0.25- and 0.5-mm precoated silica gel 60 glass sheets (5 × 10 cm, 20 × 20 cm, Merck) with *n*-hexane-ethyl ether (1 : 1). Detection of the isolated spots was performed by spraying with 50% $H_2SO_4$ followed by heating.

*Gas Chromatograph–Mass Spectrometry (GC-MS)*. GC-MS results were recorded with a JMS D-100 GC-MS. Ionization voltage was 25 eV and ion source temperature was 200° C. A glass column (3 mm × 2 m) coated with OV-1 3% on Chromosorb WAW was used. The injection port temperature was kept at 250° C. Helium was used as a carrier gas at an inlet pressure of 1.0 kg/cm$^2$.

*Preparation of Pellets Used for Oviposition Assay*. The pellets (9 mm in diameter, 4 mm thickness, hardness 2.5 kg/cm$^2$, weight 210 mg) used in the assay were kindly prepared from cornstarch at the pharmacy of Hiroshima University Hospital. The pellets were steeped with the sample solution (0.2 mg/ml, 2 mg/ml, 20 mg/ml) for 10 min and dried. The concentration of the sample was equivalent to 0.01 mg, 0.1 mg, and 1 mg/pellet. The thresholds of response for oviposition activity was 0.1 mg of sample per pellet.

*Oviposition Preference Assay*. Oviposition bioassay procedures (Arakaki and Takahashi, 1982) are briefly described here. Four pellets (two pellets steeped with samples and two intact pellets as control) and an adult female aged two or three weeks after emergence were placed in polystyrene vial (2.4 cm in diameter, 5 cm high, 22 ml volume) covered with 60-mesh wire net (1 cm in diameter). The assay was performed for two days under the following conditions: temperature 30° C, 72% relative humidity, illumination 18 hr light 6 hr dark. After two days, eggs deposited on the four pellets were counted by means of microscope. Each experiment was repeated six times.

*Oviposition Activity Index (OAI)*. The preference activity of a sample is

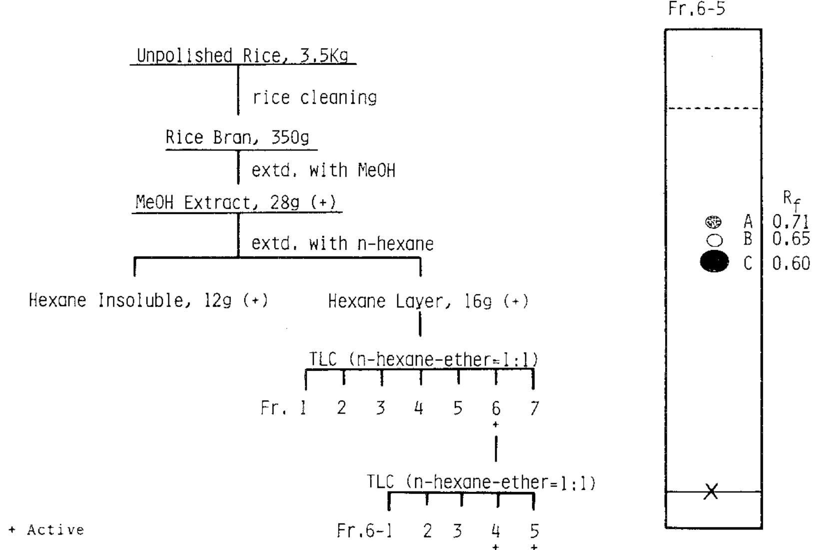


FIG. 1. Isolation of oviposition stimulants from rice bran.

expressed as the oviposition activity index (OAI) and is calculated as follows (Kramer and Mulla, 1979): OAI = $(N_s - N_c)/(N_s + N_c)$. $N_s$ denotes the number of deposited eggs in treated pellets, and $N_c$ the number of eggs in control pellets. All index values lie within the range +1 to −1. Positive values indicate that more ovipositions were observed in the treated pellet than the control, and conversely more ovipositions in the control than in the treated pellet result in a negative OAI. The data were analyzed statistically, and the significance of all indices was determined using chi-square analysis.

*Isolation of Active Substances.* The isolation procedure is shown in Figure 1. Rice bran (350 g) obtained from unpolished (brown) rice (3.5 kg) of the variety Nakate-Sinsenbon by rice cleaning was extracted with methanol. The methanol extract (28 g) which exhibited positive oviposition response was then extracted with *n*-hexane to give a hexane layer (16 g) and a hexane-insoluble portion (12 g). Then the hexane extract was separated into seven fractions by preparative TLC using *n*-hexane–ethyl ether (1:1). Fraction 6 (980 mg), which exhibited positive oviposition response, was separated by the same TLC system into five fractions. Fraction 6-4 (333 mg) and Fraction 6-5 (59 mg), consisting of three components [$R_f$ = 0.71 (A), 0.65 (B), 0.60 (C)] on TLC as shown in Figure 1 exhibited positive responses, and the three components [A (55 mg), B (50 mg), C (210 mg)] were isolated by preparative TLC using *n*-hexane–ethyl ether (1:1).

## RESULTS

*Identification of Components A, B, and C.* The infrared (IR) spectrum of component A showed the presence of aromatic moieties (1605, 1597, 1517 $cm^{-1}$) and conjugated ester carbonyl (1705 $cm^{-1}$ shifted to 1722 $cm^{-1}$ by hydrogenation). Hydrolysis of component A with 10% KOH in MeOH gave ferulic acid (identified by mixture mp and IR spectrum 3470, 1695, 1680, 1620, 1600, 1590, 1520, 1280, 1205, 1115 $cm^{-1}$) from the acidic fraction and 24-methylenecycloartanol [identified by GC-MS as shown in Figure 2, *m/z* 440($M^+$), 422, 407, 379, 353, 313, 300, 285, 203, 175], cycloartenol [*m/z* 426($M^+$), 411, 408, 393, 365, 339, 315, 286, 271, 217, 203, 189, 175], sitosterol [*m/z* 414($M^+$), 396, 381, 329, 303, 273, 255, 231, 213], and campesterol [*m/z* 400($M^+$), 382, 367, 340, 315, 289, 273, 231, 213] from the neutral fraction. The high-resolution MS of component A showed the characteristic ferulate moiety at *m/z* 177.052 ($C_{10}H_9O_3$) together with molecular ions at 616.449 ($C_{41}H_{60}O_4$) and 602.427 ($C_{40}H_{58}O_4$) which showed 24-methylenecycloartanyl and cycloartenyl ferulates. On the basis of these results, component A was shown to be the ferulates of the triterpene alcohols and sterols (Endo et al., 1968).

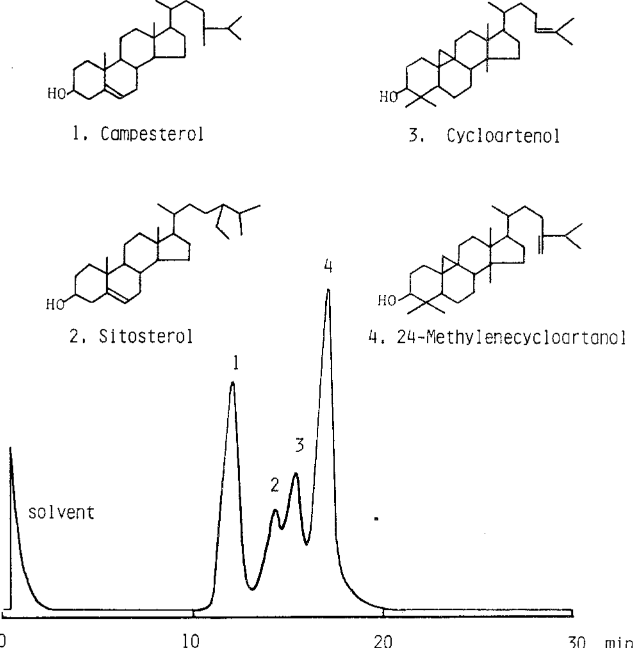


FIG. 2. Gas chromatogram of the neutral fraction obtained by hydrolysis of component A.

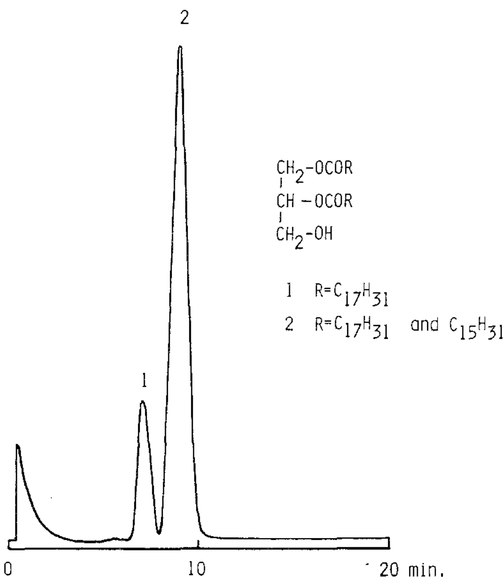


FIG. 3. Gas chromatogram of the trimethylsilyl derivative of component B.

The IR spectrum of component B showed the presence of a hydroxyl group (3500 $cm^{-1}$) and an ester carbonyl (1738 and 1600 $cm^{-1}$) in the molecules. Hydrolysis of the component with 5% KOH-MeOH gave palmitic and linoleic acids, whose methyl esters were identified by GC-MS from the acidic fraction, and glycerol, whose triacetyl derivative was identified by GC-MS from the neutral fraction. The GLC of the trimethylsilyl derivative of component B showed two peaks (Figure 3), the MS of which showed the characteristic ions at *m/z* 649 ($M^+ - 15$, consisting of palmitic and linoleic acids) and 673 ($M^+ - 15$, consisting of linoleic acid) showing the diglycerides of palmitic and linoleic acids. 1,2-Disubstitution of the diglycerides was confirmed by the presence of [$^1$H]NMR signals of the monoacetate of the diglycerides at 2.08 (OAc) and 4.27 (2H, m, —$\underline{CH_2}$—OAc), and by the absence of the ions of $(M—C_{15}H_{31}COOCH_2)^+$ and $(M—C_{17}H_{35}COOCH_2)^+$ in the MS due to 1,3-disubstitution. From these results component B was shown to be 1,2-disubstituted diglycerides consisting of palmitic and linoleic acids.

The IR spectrum of component C (3640, 1465, 1375, 1040, 950 $cm^{-1}$) is very similar to that of sitosterol. Its gas chromatogram (Figure 4) showed three peaks which were identified as campesterol [*m/z* 400 ($M^+$)], stigmasterol [412 ($M^+$)], sitosterol [414 ($M^+$)] by GC-MS.

*Oviposition Assay.* When polished rice and unpolished rice were presented, rice weevils deposited eggs on unpolished rice rather than polished

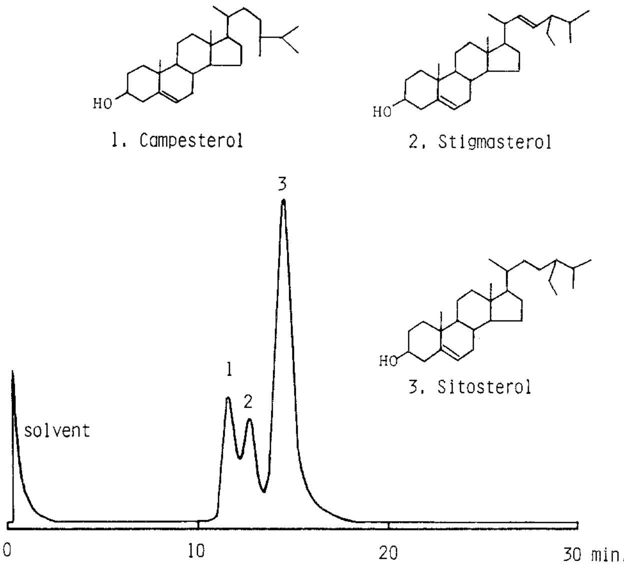


FIG. 4. Gas chromatogram of component C.

rice. When methanol extract of rice bran was added to pellets of cornstarch, the rate of oviposition was higher than when no extract was presented. The methanol extracts of rice bran from unpolished rice therefore contained oviposition stimulants. The active portions of the methanol extracts were extracted by *n*-hexane (OAI + 0.50) (Table 1 and Figure 5). From the hexane extracts, components A, B, and C were isolated by preparative TLC. The oviposition preference test of component A exhibited slightly positive responses (OAI, 0.09) (Table 1, Figure 5). On the other hand, both components B and C exhibited negative OAI activity (−0.08 and −0.15, respectively). The individual components (24-methylenecycloartanyl, cycloartenyl, sitosteryl, campesteryl ferulates, campesterol, stigmasterol, sitosterol, and 1,2-dilinoleic and 1,2-dipalmitic glycerides) obtained commercially showed no oviposition activity. We assumed that the individual components have no oviposition-stimulating activity. OAI index of the mixture of two of components A and B, B and C, C and A was −0.18, −0.05, and −0.22, while OAI of a mixture of three components A, B, and C in the original content (1:1:4) was +0.33, showing the positive response as shown in Table 1 and Figure 5.

## DISCUSSION

Arakaki and Takahashi (1982) reported that the oviposition stimulants of rice weevils are mainly in the embryonic part and aleurone layer of rice

TABLE 1. OVIPOSITION PREFERENCE FOR PELLETS MADE OF CORNSTARCH TO WHICH COMPONENTS A, B, AND C WERE ADDED[a]

| | Numbers of eggs deposited on pellets | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment | H | R | A | R | B | R | C | R | A-B | R | A-C | R | B-C | R | A-B-C | R |
| 1 | 9 | 1 | 5 | 11 | 3 | 4 | 6 | 4 | 4 | 5 | 7 | 6 | 7 | 5 | 9 | 3 |
| 2 | 9 | 6 | 7 | 7 | 4 | 4 | 7 | 2 | 3 | 3 | 3 | 2 | 4 | 8 | 5 | 2 |
| 3 | 10 | 6 | 10 | 2 | 6 | 7 | 3 | 5 | 4 | 3 | 7 | 11 | 4 | 6 | 10 | 5 |
| 4 | 12 | 0 | 9 | 6 | 8 | 7 | 2 | 6 | 1 | 4 | 5 | 6 | 7 | 9 | 11 | 6 |
| 5 | 8 | 5 | 6 | 7 | 6 | 8 | 1 | 7 | 2 | 2 | 1 | 11 | 6 | 7 | 4 | 1 |
| 6 | 9 | 1 | 10 | 6 | 3 | 5 | 4 | 7 | 2 | 6 | 4 | 6 | 5 | 5 | 3 | 4 |
| Total | 57 | 19 | 47 | 39 | 30 | 35 | 23 | 31 | 16 | 23 | 27 | 42 | 36 | 40 | 42 | 21 |
| $\overline{X}$ | 9.50 | 3.17 | 7.83 | 6.50 | 5.00 | 5.83 | 3.83 | 5.17 | 2.65 | 3.83 | 4.50 | 7.00 | 6.00 | 6.67 | 7.00 | 3.50 |
| SD | 1.37 | 2.79 | 2.14 | 2.88 | 2.00 | 1.72 | 2.31 | 1.92 | 1.21 | 1.47 | 2.35 | 3.46 | 1.26 | 1.63 | 3.41 | 1.87 |
| SE | 0.56 | 1.14 | 0.87 | 1.18 | 0.82 | 0.70 | 0.95 | 0.79 | 0.49 | 0.60 | 0.96 | 1.41 | 0.52 | 0.67 | 1.39 | 0.76 |
| $\chi^2$ | 19.000 | | 0.744 | | 0.385 | | 1.185 | | 0.126 | | 3.261 | | 0.211 | | 7.000 | |
| OAI | 0.50 ± 0.11 | | 0.09 ± 0.15 | | −0.08 ± 0.14 | | −0.15 ± 0.19 | | −0.18 ± 0.18 | | −0.22 ± 0.21 | | −0.05 ± 0.09 | | 0.33 ± 0.18 | |

d.f = 1 $\chi^2_{0.05} = 3.841$

[a]H: hexane extract, A: component A was added, B: component B, C: component C, A-B: components A and B were added, A-C: components A and C, B-C: components B and C, A-B-C: components A, B, and C were added, R: control, OAI: oviposition activity index.

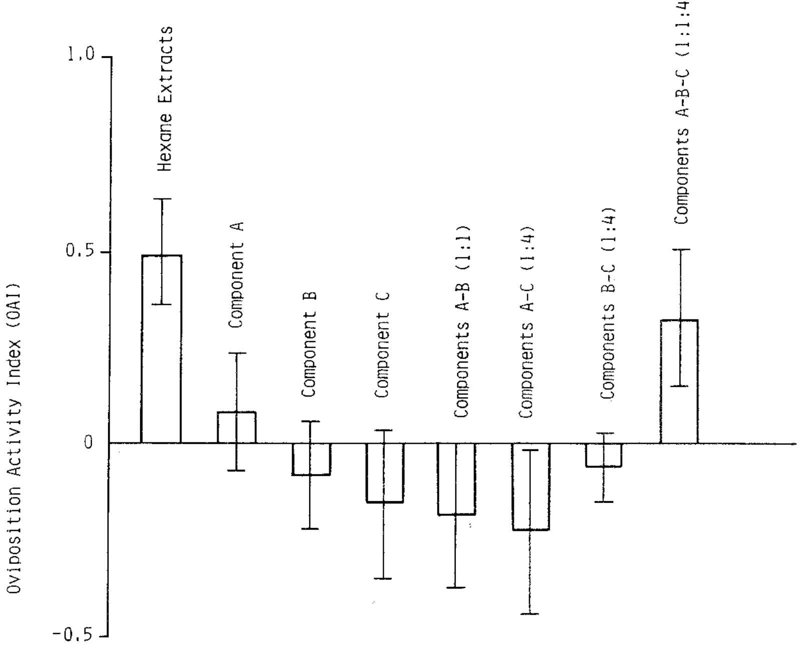


FIG. 5. Oviposition activity index of *n*-hexane extract and components A, B, and C.

grain. The *n*-hexane-soluble portion of the methanol extracts of rice bran contained *n*-paraffins, steryl esters, alkyl esters, glycerides, fatty acids, ferulates, and sterols, in which a certain mixture of ferulates, diglycerides, and sterols exhibited oviposition activity. The individual components of the stimulants showed a repellent activity when presented singly or in combinations of two. Accordingly, we concluded that the oviposition preference for the rice weevil can be induced by the synergistic action of the three components. Nishida (1977) reported similar synergistic action of the oviposition stimulants of *Byasa alcinous*. In addition to the oviposition stimulants of the weevils, starch was required as the pellet construction. The weevil deposited eggs on the pellets made of starch rather than cellulose or silica gel. A solid shape of grain is needed for the oviposition but is not necessary for feeding. When the pellet containing the stimulant was ground into powder, the weevils did not deposit eggs on starch flour but did it on the solid pellets made of powdered starch (Arakaki and Takahashi, 1982). The shape and size of the pellet have no effect on the oviposition preference, since the weevils deposited eggs on rice grain and tablets equally.

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(Faculty of Science, Hiroshima University) for the high-resolution MS spectra of ferulates. Mr. N. Arakaki (Faculty of Integrated Arts and Sciences, Hiroshima University) provided several useful suggestions for the bioassay.

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# TRAP TREES FOR ELM BARK BEETLES
## Augmentation with Pheromone Baits and Chlorpyrifos

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**Abstract**—European elm bark beetles, *Scolytus multistriatus* (Marsh.), were strongly attracted to American elms, *Ulmus americana* L., baited with the *S. multistriatus* attractant, multilure, or killed by injection of the arboricide, cacodylic acid; a combination of the two treatments was most attractive. Comparisons of beetle catches on sticky bands affixed to the trees with samples of bark from the same trees showed that the number of beetles landing on cacodylic acid-treated trees was approximately 40 times greater than the number boring into them. Spraying the bark with the insecticide chlorpyrifos had no direct effect on attraction. No live bark beetle brood was found in trees that had been treated with cacodylic acid or chlorpyrifos, but trees that were only baited or left untreated (check) were attacked, killed, and colonized. We suggest that the contribution of the cacodylic acid trap tree technique to Dutch elm disease control will be enhanced by baiting treated trees with multilure and spraying their lower boles with 0.5% chlorpyrifos. This treatment will eliminate diseased and unwanted elms as potential breeding material and kill large numbers of elm bark beetles that might otherwise innoculate healthy elms with the Dutch elm disease fungus.

**Key Words**—European elm bark beetle, *Scolytus multistriatus*, Coleoptera, Scolytidae, control, chlorpyrifos, cacodylic acid, trapping, trap tree, Dutch elm disease, multilure, attraction.

## INTRODUCTION

The fungus [*Ceratocystus ulmi* (Buisman) C. Moreau] that causes Dutch elm disease (DED) is spread by elm bark beetles [*Scolytus multistriatus* (Marsh.) and *Hylurgopinus rufipes* (Eichh.)] that breed in moribund elms



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and feed in the twigs and branches of healthy trees. (For reviews of DED and elm bark beetles see Sinclair and Campana, 1978; Stipes and Campana, 1981; Kondo et al., 1982). A principal measure for controlling DED is sanitation—the removal and destruction of potential breeding material. However, this approach is constrained by its expense and the brevity of the period during which the work must be completed.

O'Callaghan et al. (1980) developed a trap tree technique for expediting and augmenting sanitation for DED control. Hopelessly diseased or unwanted elms are killed by injection with cacodylic acid ("CACing"). CACd trees are very attractive to native (*H. rufipes*) (Gardiner, 1979) and European (*S. multistriatus*) (Lanier, unpublished data; O'Callaghan, 1984) elm bark beetles; attraction of the latter species may be enhanced by baiting the tree with multilure, a synthetic copy of its aggregation pheromone (Pierce et al., 1975; Lanier et al., 1976).

Both species of bark beetles attempt to colonize CACd trees, but development of their broods is inhibited by aboricide-induced drying of the bark. In a trial in Washington, D.C. (Lanier, 1982), development of *S. multistriatus* broods was reduced by 98% in trees in which at least $\frac{1}{2}$ of the crown was alive and by 52% in trees that had $\frac{1}{10}$ or less of the crown alive at the time of treatment.

Owing to their minimal contribution to the next beetle generation, CACd shade trees can be removed at a convenient schedule, and those in green spaces can be left standing; there is no need to burn or bury the wood. Because the treatment is quick and inexpensive (0.2–1 man-hour per tree and $0.25–$10 for cacodylic acid; Lanier, unpublished data), and great economies in removal of trees can be realized, the method is a cost-powerful alternative to conventional sanitation.

In addition to eliminating diseased elms as breeding material, the trap tree concept seeks to reduce current year DED infections by attracting bark beetles that might otherwise feed in healthy trees. Millions of beetles were absorbed by trap trees and DED rates declined sharply in several areas where the technique was extensively employed (O'Callaghan et al., 1980; Lanier, 1982).

In England, O'Callaghan et al. (1984) found that CACd elms attracted many more elm bark beetles [*Scolytus scolytus* (L.), *S. multistriatus*] than bored into them. In New York it had been noted (O'Callaghan and Lanier, unpublished) that the period during which *H. rufipes* was attracted considerably exceeded the period during which they bore into CACd trees; drying of the bark and colonization of the phloem by saprophytic fungi [similar to that noted by Webber (1981)] apparently caused many of the beetles to reject these trees as host material.

Since *S. multistriatus* land on diseased or baited elms predominantly below 4 m (Cuthbert and Peacock, 1975), we reasoned that the impact of trap

trees on the beetle population could be increased considerably by treating the lower bole with a toxicant. In tests of several insecticides for this purpose, chlorpyrifos emerged as the compound of choice; a 10-sec exposure on bark sprayed with 2% chlorpyrifos killed 90% or more of the *H. rufipes* and *S. multistriatus* six weeks after the bark was sprayed, while 1-min exposures were lethal to nearly 100% of the beetles throughout the 10-week experiment (Lanier et al., 1984).

In this paper we report the effect of CACing, baiting, and chlorpyrifos treatments on attraction, host attack, and apparent mortality of *S. multistriatus*. We reiterate the effectiveness of trap trees against elm bark beetles and show that the impact on the beetle population can be enhanced by the careful use of toxicants.

## METHODS AND MATERIALS

Sixteen trees were used in this experiment—eight within each of the two plots in Syracuse, New York. Plot A was an open field situation with small trees ranging from 3 to 20 cm diameter at breast height (dbh, 1.4 m). These trees were marked and treated May 18, 1983. Plot B was a closed-canopied stand of American elms in which trees 11.5–23 cm dbh were treated June 23, 1983.

In each plot seven trees were treated; the remaining tree was an experimental control. Treatments with cacodylic acid, chlorpyrifos, and multilure bait were administered in all singlet, doublet, and triplet combinations (Table 1). For the CAC treatments, undiluted cacodylic acid (Rad-E-Cate 35®, Vineland Chemical Co., Vineland, New Jersey, 27.4% active ingredient was applied from a pressurized sprayer to the point of runoff into an axe frill approximately 65 cm above ground. The amount of herbicide used ranged from approximately 10 ml for an 8-cm dbh tree to 50 ml for a 23-cm dbh tree. For the insecticide treatments, the contact insecticide chlorpyrifos (Dursban 2E®, Dow Chemical Co., Midland, Michigan, 0.5% aqueous solution) was sprayed to runoff on tree boles from ground level to 4 m. To bait trees, aggregation pheromone of *S. multistriatus* was released from a laminated plastic Hercon® (Health-Chem Corp., Plainfield, New Jersey) dispenser nailed to tree boles at approximately 2.5 m. The multilure bait was designed to last 90–120 days and to release the following nominal daily amounts of its three components: 25 $\mu$g of 4-methyl-3-heptanol, 6 $\mu$g $\alpha$-multistriatin, and 50 $\mu$g of $\alpha$-cubebene. Baits used provided one quarter of the output of lures used on sticky traps for mass trapping *S. multistriatus* (Lanier, 1981; Peacock et al., 1981).

To monitor insect activity, each tree was banded at 0.5- and 2.3-m heights with 30-cm-wide clear polyethylene sheets coated with a sticky substance.

Beetles were removed from sticky bands weekly until August 18. An additional collection was made on September 2 and a final one on October 10

TABLE 1. *Scolytus multistriatus* ON STICKY BANDS AND ATTACKING ELM TREES IN PLOTS A AND B (SYRACUSE, NEW YORK, MAY 18–OCT. 10, 1983)

| Treatment[a] | Plot | Diam. (cm) | Percent of total beetles caught in flight periods 1, 2, and 3[b] | | | | Attacks[c] | | Final condition of tree |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | Total | Density | % Success | |
| C-S-B | A | 9.0 | 16.9 | 9.3 | 2.0 | 9.6 | 0 | — | Dead |
| | B | 16.5 | 27.6 | 35.9 | 23.1 | 29.2 | 0.60 | 0 | Dead |
| C-S | A | 4.0 | 14.3 | 6.9 | 1.5 | 7.9 | 0 | — | Dead |
| | B | 14.5 | 9.6 | 6.3 | 8.8 | 7.9 | 0 | — | Dead |
| C-B | A | 5.1 | 35.3 | 6.5 | 1.9 | 17.3 | 0 | — | Dead |
| | B | 16.5 | 33.5 | 19.9 | 17.8 | 21.2 | 1.15 | 0 | Dead |
| S-B | A | 20.3 | 11.8 | 57.3 | 38.0 | 29.2 | 1.32 | 0 | Live |
| | B | 14.0 | 7.3 | 8.6 | 17.5 | 12.1 | 0.06 | 0 | Live |
| C | A | 8.5 | 14.6 | 6.2 | 1.1 | 7.8 | 0 | — | Dead |
| | B | 11.5 | 12.3 | 21.1 | 13.8 | 16.7 | 1.93 | 0 | Dead |
| S | A | 3.0 | 0.2 | 2.9 | 1.2 | 1.0 | 0.57 | 0 | Live |
| | B | 15.0 | 0.6 | 0.5 | 0.7 | 0.6 | 0 | — | Live |
| B | A | 8.5 | 6.8 | 6.9 | 24.9 | 14.3 | 8.89 | 0*[d] | Dead |
| | B | 14.0 | 9.0 | 7.6 | 10.4 | 9.0 | 2.89 | 100 | Dead |
| Control | A | 6.4 | 0.1 | 4.0 | 29.4 | 12.8 | 3.23 | 0* | Dead |
| | B | 23.0 | 0.1 | 0.1 | 7.9 | 3.3 | 8.13 | 54.6 | Dead |
| Total beetles | A | | 2804 | 917 | 6323 | | | | |
| | B | | 2612 | 6866 | 16384 | | | | |

[a]C, cacodylic acid applied in axe frill; S, sprayed with 0.5% chlorpyrifos to height of 3 m; B, baited with multilure.
[b]Periods 1, 2, and 3 conform to the principal flight periods in Syracuse, New York. May 18–July 13, first generation; July 14–August 18, second generation; August 19–October 10, partial 3rd generation (most progeny of second generation overwinter as larvae).
[c]Attacks/$dm^2$ (density) and percent producing larvae (success).
[d]Trees (*) killed with cacodylic acid soon after colonization began; broods would have been expected to survive had the trees not been treated.

when trees were felled. Bolts 10 cm long were cut from these trees immediately above each of the two sticky bands. Bark beetle colonization was ascertained by removing the bark from the samples to record the number of attacks (penetrations to xylem) and the number of female galleries with larvae (brood success).

## RESULTS AND DISCUSSION

### *Bark Beetle Activity in Plots*

Very few *H. rufipes* were captured on sticky bands on any of the experimental trees, and no galleries of this species were found in the tree sections sampled. Therefore, only *S. multistriatus* is considered further in our discussions.

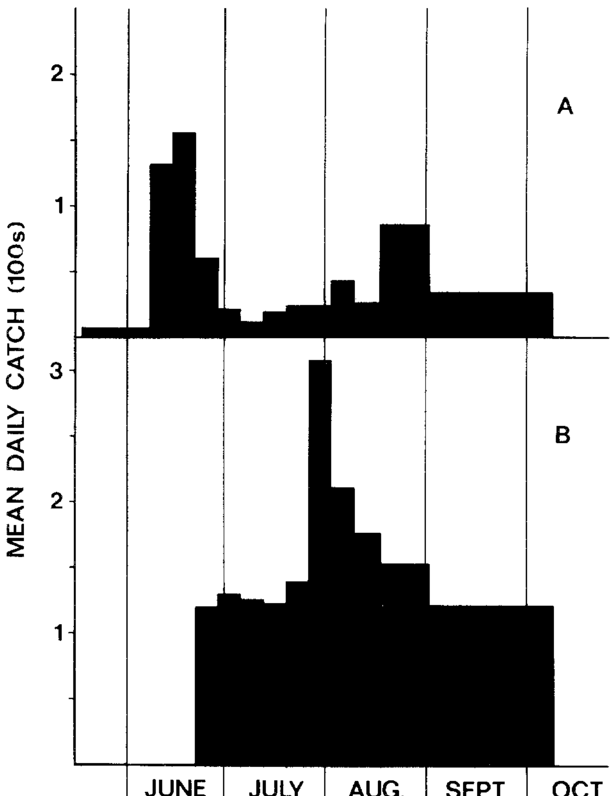


FIG. 1. Mean daily catch of *Scolytus multistriatus* on sticky bands in experimental plots A and B. From June through mid August collection intervals (represented by column widths) were generally seven days; those preceding and succeeding ranged from 14 to 36 days. Trees in plot A treated May 18, 1983; trees in plot B treated June 23, 1983.

The population of European elm bark beetles in the study area was extremely large, owing to abundant breeding material created by elm yellows disease (elm phloem necrosis). Totals of 6323 and 16,384 European elm bark beetles were taken on sticky bands on trees in plots A and B, respectively (Table 1). Assuming the beetles landed randomly over the entire bark surface of the trees, we can extrapolate from the samples that totals of approximately 58,000 and 116,000 visited the experimental trees in the respective plots. The twofold difference in numbers of beetles taken at the two plots is probably a result of the larger size and the consequent longer retention of attractiveness by the trees in plot B.

Figure 1 illustrates the catch of beetles over time. The histogram for plot A features the emergence of the spring generation (late May to mid July) and

plot B reflects the activity of the summer generation (mid July through August). A late peak (end of August) in activity on plot A is a result of the fall (partial 3rd) generation mass attacking three of the four trees that had not already been killed with cacodylic acid.

*Attractiveness of Treatments*

*Chlorpyrifos Sprays.* Figure 2 indicates that chlorpyrifos did not attract or repel *S. multistriatus;* catches on sticky bands on spray-baited trees (dark circles) moved with those on baited trees (open circles) and catches on sprayed trees (dark triangles) moved with those on control trees (open triangles) until

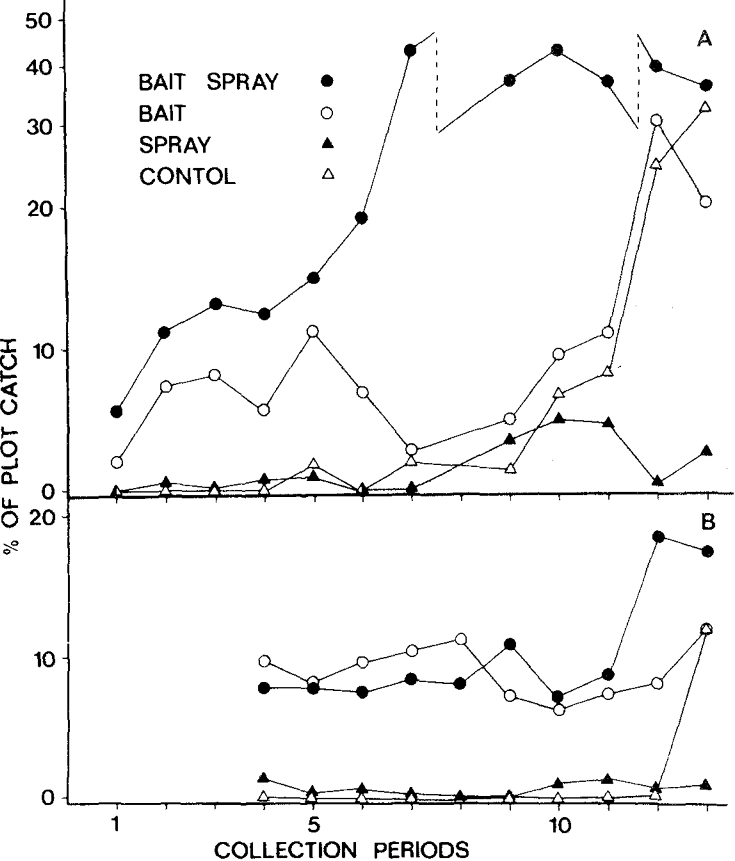


FIG. 2. *Scolytus multistriatus* taken on sticky bands of variously treated trees expressed as a percent of total plot catch for the collection period (See Figure 1). Plot A (upper) and plot B (lower). Late season increases in catches on baited and control trees reflect mass attack and colonization of these trees.

some of the trees came under mass attack (reflected by a dramatic increase in beetles captured) and natural pheromones and kairomones increased attraction. Chlorpyrifos may have had an indirect effect on attraction by reducing attack and, therefore, the production of natural pheromone. The lowest total catches in both plots were on trees that had been sprayed but not CACd or baited (Table 1, Figure 2). In contrast, the untreated check trees and baited unsprayed trees in both plots were mass attacked and killed during September. Drought stress or presymptomatic elm yellows disease, or a combination of these, apparently rendered the trees susceptible to colonization. None of the trees showed symptoms of DED.

*CAC and Baiting.* CACing or baiting caused trees to be attractive to beetles; a combination of these two treatments was usually most potent (Figure 3). On both plots, trees that were CACd and baited were initially considerably more attractive than trees that were CACd without baiting. This difference diminished (plot B) or disappeared (plot A) as baits became less potent. Our experience with the Hercon baits indicated that attractiveness of baits should have endured the entire summer. It seems possible that the additive effect of pheromone baits on attraction to CACd trees was diminished by

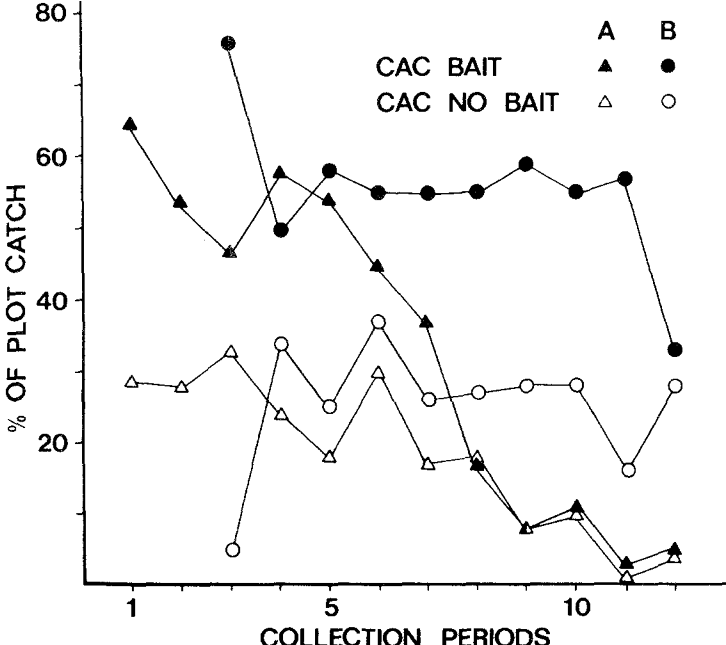


Fig. 3. *Scolytus multistriatus* taken on sticky bands on CACd and baited trees vs. CACd unbaited trees.

competition with natural pheromones from many trees within the plots that were being attacked by *S. multistriatus* during late summer.

*Attack and Colonization of Trees*

*Effect of CACing.* Cacodylic acid treatment reduced attack density and precluded brood development (Table 2). The CACd elms in plot A were devoid of colonization attempts. We assume that the small size (4–9 cm diam.) and early treatment (May 18) combined to make these trees to dry for *S. multistriatus* to accept for colonization. Three of the four trees CACd in plot B were attacked, but attraction exceeded attacks by a factor of 38.6. In contrast, attraction was estimated to be only 3.4–8.2 times greater than attacks on non-CACd trees (Table 2). The CAC treatment causes the bark to dry, and we have observed massive colonization of bark of treated trees by saprophytic microorganisms that compete with bark beetles for nutrients, as was reported to occur in England by O'Callaghan et al. (1984). Therefore, the treatment must be closely coordinated with beetle flight periods if the number of beetles absorbed by CACd trees is to be maximized.

*Effect of Chlorpyrifos Sprays.* Sprayed trees were either not attacked or the attacks were unsuccessful because the beetles died before they oviposited. The only trees that survived to the end of the experiment were those receiving the spray and the spray-bait treatments; this indicates that the insecticide provided protection from attack. Baited–unsprayed and check trees in both plots were killed and colonized. Interruption of colonization on sprayed trees was expected because Lanier et al. (1984) found that *S. multistriatus* was unable to colonize chlorpyrifos-treated bark even 20 weeks after treatment.

TABLE 2. ATTRACTION–ATTACK INDICES FOR CACD AND NON CACD ELM TREES (SYRACUSE, NEW YORK, MAY 18–OCT. 10, 1983)

| Treatment[a] | Plot | Beetles trapped[b] | Beetle attacks[c] | Attraction–attack[d] index |
|---|---|---|---|---|
| CACd | A | 2,707 | 0 | |
| | B | 12,266 | 53 | 38.6 |
| nonCACd | A | 3,616 | 74 | 8.2 |
| | B | 4,118 | 202 | 3.4 |

[a]CACd includes C-S-B, C-S, C-B, and C; nonCACd includes S-B, S, B, and check (see Table 1).
[b]Total *S. multistriatus* collected on two 30-cm-wide circumferencial sticky bands at 1-m and 2.1-m heights.
[c]Total penetrations by *S. multistriatus* to the cambial region in two 10-cm-long bole sections cut approximately 1.5-m and 3-m heights.
[d]$I = (t/asr)$ where $I$ is the index, $t$ = trap catch, $a$ = number of attacks, $s$ = 2 (one male + one female)/attack and $r$ = 3 (sticky band size over bark sample size. Thus $I = (t/a \times 6)$.

## CONCLUSIONS

Elm trees that are killed with cacodylic acid or baited with multilure are very attractive to *S. multistriatus*. The combination of these two measures is most attractive. Chlorpyrifos had no direct effects on beetle attraction.

The number of elm bark beetles eliminated by the cacodylic acid-trap tree technique could be greatly increased by spraying the CACd trees with 0.5% chlorpyrifos. This compound is extremely effective against elm bark beetles (Gardiner and Webb, 1980; Lanier et al., 1984) and, as Dursban 2E® and Dursban 4E® (Dow Chemical), it is registered for use on boles of elms. Application of the insecticide to a height of 4 m on the tree bole would be effective because *S. multistriatus* generally land below this level (Cuthbert and Peacock, 1975). Such treatments can be accomplished with simple manually operated sprayers, although commercial equipment would reduce time required and increase the amount of bark surface covered.

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# RESPONSE OF NORTHERN CORN ROOTWORM, *Diabrotica barberi* SMITH AND LAWRENCE,[1] TO STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE[2]

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**Abstract**—The four stereoisomers of 8-methyl-2-decyl propanoate were tested in South Dakota for attractiveness to the northern corn rootworm, *Diabrotica barberi* Smith and Lawrence (NCR). Only the 2*R*,8*R* configuration was attractive to the NCR. Inhibition of the NCR response to 2*R*,8*R* occurred when either the 2*S*,8*R* or 2*S*,8*S* isomers were components of the pheromone source. The 2*R*,8*S* configuration elicited no behavioral activity in the NCR.

**Key Words**—Chrysomelidae, *Diabrotica barberi*, northern corn rootworm, sex pheromone, stereospecificity, inhibition, enantiomer.

## INTRODUCTION

The northern corn rootworm, *Diabrotica barberi* Smith and Lawrence (NCR), is a common pest of corn east of the Rocky Mountains in both the U.S. and Canada. The NCR is often found cohabiting with the western corn rootworm, *D. virgifera virgifera* Le Conte (WCR), with which it shares a similar life history (Branson and Krysan, 1981).

[1]Coleoptera: Chrysomelidae.
[2]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.
[6]Deceased.

Until recently, widespread uncertainty existed as to whether or not the NCR and WCR were distinct species (Chiang, 1973). This confusion stemmed from frequent field observations of apparent interspecific matings, observations and collections of putative hybrids, and the reported production of hybrids in the laboratory (Hintz and George, 1979). Notwithstanding the latter, Krysan and Guss (1978) showed that, although hybridization between the NCR and WCR is possible under laboratory conditions, reproductive barriers between the two species exist to the extent that hybridization under natural conditions is unlikely.

It is well documented that females of the NCR and WCR are cross-attractive to males of either species. Guss (1976) showed that extracts from virgin WCR females attracted males of both the WCR and NCR, although a temporal difference in response was demonstrated, i.e., WCR males responded during daylight hours while NCR males responded at night. These observations were confirmed by Bartelt and Chiang (1977), who further showed that virgin females of both species attracted males of either species to baited traps.

The sex pheromone of the WCR was identified as 8-methyl-2-decyl propanoate (Guss et al., 1982), and subsequent studies indicated that the configuration of the naturally produced pheromone was 2*R*,8*R* (Guss et al., 1983). At low doses, racemic 8-methyl-2-decyl propanoate was attractive to males of the NCR, but at loadings of 10 $\mu$g or more, dispensed from rubber septa, the response of NCR males to the synthetic racemate was extinguished (Guss et al., 1982). This result suggested, among other possibilities, that one or more of the stereoisomers in racemic 8-methyl-2-decyl propanoate was inhibiting the response by male NCR to the active component(s).

We have recently reported on the response of the WCR and two other *Diabrotica* to the individual stereoisomers of 8-methyl-2-decyl propanoate (Guss et al., 1983. This report deals with the response of the NCR to those same isomers.

## METHODS AND MATERIALS

The stereoisomers used in this study are the same preparations used earlier (Guss et al., 1983). Syntheses of these components were accomplished by a convergent approach in which two fragments, each containing one asymmetric center, were joined to complete the required sequence. The configurational purity of each fragment, which assured the configurational purity of the final product, was determined absolutely by GLC and/or HPLC using diastereomeric derivatives (Sonnet and Heath, 1982; Carney et al., unpublished). Isomeric purity of the target isomers was: 2*R*,8*R* (97.8%); 2*R*,8*S* (98.3%); 2*S*,8*R* (97.4%); and 2*S*,8*S* (98.8%).

The individual isomers, or specific mixtures, were diluted to appropriate concentrations in hexane and dispensed into the "cup" portion of rubber septa (A.H. Thomas No. 8753-D22) in 50 $\mu$l quantities to produce the pheromone sources. The total amount of pheromone per source was limited to 1 $\mu$g when testing individual isomers or 3.5 $\mu$g in mixtures, to obviate as much as possible effects attributable to the small amounts of nontarget isomers in these preparations.

Pheromone traps were constructed from plastic-coated milk carton blanks, and, when deployed, were the shape of a vertically oriented triangular prism 9 × 20 cm on each face. The traps, coated on the outside with Tangle-Trap, were placed on wooden stakes (1 m) constructed from lathing (1.0 × 3.5 cm) and equipped with a 20-cm-long crossbar 20 cm from the top. To ensure that the sticky surfaces of the traps did not lose their trapping efficiency because of accumulation of dirt and debris, fresh traps were placed on the stakes daily in all experiments with one exception. In the third test (Table 3) traps were deployed for five days and changed four times (24 hr, 24 hr, 24 hr, 48 hr). Pheromone sources were attached to the tops of the stakes with a No. 4 insect pin and were approximately first ear height. Distance between traps was approximately 25 m. All tests were conducted in cornfields near Brookings, South Dakota, in July and August 1982.

## RESULTS AND DISCUSSION

Response by NCR males to the individual stereoisomers is shown in Table 1. Male NCR were attracted only to those traps baited with the 2*R*,8*R* isomer; this is the same configuration preferred by WCR males, and probably the only configuration produced by WCR females (Guss et al., 1983). This result is consistent with earlier work that showed that NCR males are attracted

TABLE 1. RESPONSE BY *D. barberi* MALES TO STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE

| Isomer (1.0 $\mu$g) | Mean No. NCR/trap ± SD[a] |
|---|---|
| 2*R*,8*R* | 123.5 ± 48.2 a |
| 2*S*,8*R* | 2.0 ± 1.4 b |
| 2*R*,8*S* | 5.7 ± 3.6 b |
| 2*S*,8*S* | 4.7 ± 3.0 b |
| Solvent blank | 5.7 ± 3.8 b |

[a]Four traps for each treatment were deployed for two days. Total beetle counts for each trap for two days were pooled. Means followed by the same letter are not significantly different. (Duncan NMRT, $\alpha = 0.05$, $N = 4$).

to volatiles from WCR females (Guss, 1976). The high degree of stereospecificity shown here and the fact that virgin females of both the NCR and WCR are attractive to males of both species (Bartlet and Chiang, 1977) suggest that the sex pheromone of the NCR is largely, if not exclusively, 8*R*-methyl-2*R*-decyl propanoate. In the absence of direct analysis, however, we acknowledge that other compounds may be involved.

These two species occupy the same habitat at the same time, but there is ample evidence that they have only recently become sympatric, which might account for use of the same sex pheromone (Krysan et al., 1982). In those fields in which the NCR and WCR cohabit, it is common to observe instances of apparent interspecific matings. In the vast majority of those sightings, the putative interloper is the NCR male (V.M. Kirk, personal communication). Since both species apparently use the same sex pheromone, such behavior would not be unexpected. The fact that the NCR male is far more likely to engage in interspecific encounters may reflect a lower response threshold to the pheromone. Guss et al. (1982) found that the response threshold for the NCR to racemic 8-methyl-2-decyl propanoate was approximately 10 times less than that for the WCR.

Notwithstanding frequent observations of interspecific mountings and one report that hybrids were obtained in the laboratory (Hintz and George, 1979), Krysan and Guss (1978) showed that, although hybridization between the NCR and WCR is possible, reproductive barriers exist between the two species such that hybridization in nature is unlikely. Evidence of such barriers included a very low incidence of insemination in interspecific pairings, occurrence only of conspecific insemination in competitive experiments involving males of one species and females of both species, and very low viability of eggs resulting from interspecific inseminations.

When the active isomer, 2*R*,8*R*, was mixed individually with the other three in a 1:1 ratio, inhibition of the response of the NCR to 2*R*,8*R* was observed with both the 2*S*,8*R* and 2*S*,8*S* configurations while no apparent effect was shown with 2*R*,8*S* (Table 2). These results explain our earlier finding that traps baited with relatively high levels (10 $\mu$g) of racemic 8-methyl-2-decyl propanoate do not capture significant numbers of NCR males (Guss, et al., 1982).

The relative effect of the two inhibiting isomers is shown in Table 3. As little as 0.1 $\mu$g of 2*S*,8*R* in these preparations reduced captures to essentially that of the solvent blank, whereas reduction in captures was not apparent with 2*S*,8*S* until at least 0.5 $\mu$g were present. With 2*S*,8*R*, captures significantly less than those of the solvent blank began to appear when the inhibiting isomer was present at levels of 0.5 $\mu$g or more. This would suggest that the presence of 2*S*,8*R* in relatively high levels results in actual avoidance rather than simply blocking perception by the NCR of 2*R*,8*R*.

In an earlier study, we concluded that the configuration of the natural

TABLE 2. RESPONSE BY *D. barberi* MALES TO MIXTURES OF STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE CONTAINING 2*R*,8*R* IN COMMON

| Isomer mixture | Mean No. NCR/trap ± SD[a] |
|---|---|
| 1 μg 2*R*,8*R* + — | 175.0 ± 21.6 a |
| 1 μg 2*R*,8*R* + 1 μg 2*R*,8*S* | 154.0 ± 44.3 a |
| 1 μg 2*R*,8*R* + 1 μg 2*S*,8*S* | 68.7 ± 17.7 b |
| 1 μg 2*R*,8*R* + 1 μg 2*S*,8*R* | 7.0 ± 3.5 c |
| Solvent blank | 12.0 ± 3.2 c |

[a]Four traps for each treatment were deployed for three days. Total beetle counts for each trap for three days were pooled. Means followed by the same letter are not significantly different. (Duncan NMRT, $\alpha = 0.05$, $N = 4$).

pheromone produced by the WCR was probably 2*R*,8*R*, despite the finding that the male WCR responds to both 2*R*,8*R* and 2*S*,8*R* (Guss et al., 1983). Central to our argument was the fact that males of *D. porracea* Harold respond to racemic 8-methyl-2-decyl propanoate, respond only to 2*S*,8*R* among the resolved stereoisomers, and do not respond at all to unfractioned volatiles from female WCR known to be attractive to WCR males. The other two isomers, 2*R*,8*S* and 2*S*,8*S*, were considered to be unlikely components since neither produced any discernable biological activity when tested with the WCR.

The results in the present study and those found earlier are wholly consistent with the above. Thus, Guss (1976) found that unfractionated volatiles from virgin female WCR were highly attractive to NCR males, and Bartelt

TABLE 3. EFFECTS OF VARYING LEVELS OF 2*S*,8*R* AND 2*S*,8*S* ON RESPONSE BY *D. barberi* MALES TO 8*R*-METHYL-2*R*-DECYL PROPANOATE

| | Mean No. NCR males/trap ± SD[a] | |
|---|---|---|
| Isomer mixture | 2*S*,8*R* | 2*S*,8*S* |
| 1 μg 2*R*,8*R* | 72.8 ± 15.7 a | 131.6 ± 43.6 a |
| 1 μg 2*R*,8*R* + 0.1 μg | 30.0 ± 8.5 b | 133.8 ± 33.6 a |
| 1 μg 2*R*,8*R* + 0.25 μg | 17.4 ± 7.0 cd | 113.0 ± 25.1 ab |
| 1 μg 2*R*,8*R* + 0.5 μg | 12.0 ± 1.6 de | 88.4 ± 27.2 bc |
| 1 μg 2*R*,8*R* + 1.0 μg | 6.0 ± 3.1 e | 68.6 ± 17.3 cd |
| 1 μg 2*R*,8*R* + 2.5 μg | 3.2 ± 2.8 e | 51.8 ± 24.6 d |
| Solvent blank | 25.0 ± 2.9 bc | 37.8 ± 8.1 d |

[a]Five traps for each treatment were deployed for five days. Total beetle counts for each trap for five days were pooled. Means followed by the same letter are not significantly different. (Walter-Duncan K ratio (SAS) = 100, $N = 5$).

and Chiang (1977) observed that females of both species were attractive to males of either species. Our finding in this study that males of the NCR are severely inhibited in their response to 2*S*,8*R* by the presence of low levels of 2*S*,8*R* would tend to corroborate the conclusion that 2*S*,8*R* is not a component of the natural WCR pheromone.

The inhibition of NCR attraction to 2*R*,8*R* by 2*S*,8*R* may have a role to play in reproductive isolation between NCR and *D. longicornis* (Say). These two taxa were considered to be subspecies until Krysan et al. (1983) reexamined the *longicornis* complex and elevated the NCR to species rank. We have recently found that male *D. longicornis* respond only to 2*S*,8*R* among the resolved isomers of 8-methyl-2-decyl propanoate (P.L. Guss and J.L. Krysan, unpublished data).

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# PHYTOTOXIC PROPERTIES OF NORDIHYDROGUAIARETIC ACID, A LIGNAN FROM *Larrea tridentata* (CREOSOTE BUSH)

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**Abstract**—The phytotoxic properties of nordihydroguaiaretic acid (NDGA) isolated from creosote bush, *Larrea tridentata* (Zygophyllaceae), were examined. NDGA dramatically reduces the seedling root growth of barnyard grass, green foxtail, perennial ryegrass, annual ryegrass, red millet, lambsquarter, lettuce, and alfalfa, and reduces the hypocotyl growth of lettuce and green foxtail. It has no effect on the germination of lettuce seeds. NDGA almost certainly contributes to the observed allelopathic nature of creosote bush.

**Key Words**—Allelopathy, nordihydroguaiaretic acid, NDGA, *Larrea tridentata*, creosote bush, Zygophyllaceae.

## INTRODUCTION

Creosite bush, *Larrea tridentata* Cav. (Zygophyllaceae), is the dominant shrub of the North American deserts. *Larrea tridentata* can occur in almost pure stands of up to 6000 plants/hectare (Rhoades, 1977) which suggests it may possess allelopathic properties. The soils under the closely related *L. divaricata* have been found to be water repellent, probably from substances leached from the aboveground plant parts, leading to areas nearly devoid of annual vegetation immediately under the plants (Rice, 1974). As the chemistry of *L. divaricata* and *L. tridentata* are very similar (Mabry et al., 1977), they would be expected to share this allelopathic property. Indeed, an early

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FIG. 1. Nordihydroguaiaretic acid (NDGA).

reported use of creosote bush was "Sprinkle it on a train bed and no weeds will grow" (Gergel, 1980).

Nordihydroguaiaretic acid (NDGA), a tetrahydroxy lignan (Figure 1), makes up 5–10% of the dry weight of the leaves of creosote bush (Mabry et al., 1977). NDGA possesses numerous biological activities (Oliveto, 1972). It has been reported to be active against bacteria (Oliveto, 1972; Shih and Harris, 1980), amebae (Segura, 1978), viruses (Mora and Zamora, 1982), fungi (Belmares et al., 1979), and cancer cells (Burk and Woods, 1963; Smart et al., 1969), and to possess antiherbivore properties (Rhodes, 1977). The lignan is also a well-known antioxidant (Oliveto, 1972) for fats and oils and was used in human foods for over 30 years for this purpose. However, although creosote bush has undergone extensive phytochemical investigations (Mabry et al., 1977), and the many biological activities of NDGA have been well studied (see Oliveto, 1972, and references therein), the phytotoxic properties of NDGA have not been reported. To fill this void, we have examined the phytotoxic action of NDGA against eight selected weed and crop plants.

## METHODS AND MATERIALS

NDGA was isolated from *L. tridentata* collected in the Sonoran desert of Arizona. Dried leaves of *L. tridentata* were extracted successively with Skelly F, ethyl ether, acetone, methanol, and water in a Soxhlet extractor. Chlorophyll was removed from the concentrated ethyl ether extract by column chromatography through Sephadex LH20 eluted with chloroform and chloroform–95% ethanol mixtures. NDGA was eluted with a 1:1 chloroform–95% ethanol mixture and crystallized upon concentration of the eluent. Recrystallization from methanol produced pure NDGA, mp (uncorr.) 186–187°, lit. (Waller and Gisvold, 1945) 184–185°.

*Seedling Growth Bioassay.* The effect of NDGA on root and hypocotyl length of weed and crop plants was tested at 5, 10, 20, 40, and 80 ppm NDGA in 40 ml of 0.5% agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan) in 9-cm Petri dishes. All test seeds were first germinated on 0.5% agar in a growth chamber set at 58°C, 8-hr nights, 68°C, 16-hr days. Germinated

seedlings (10–15) were transferred to the prepared Petri dishes and incubated in the dark at 21–23°C for 45–74 hr. Two controls were run: one contained only 0.5% agar, the second contained agar plus the solvent (acetone or methanol) used to dissolve the NDGA. The effect of NDGA on root growth of barnyard grass (*Echinochloa crusgalli*), green foxtail (*Setaria viridis*), perennial ryegrass (*Lolium perenne*), annual ryegrass (*Lolium multiflorum*), red millet (*Panicum miliaceum*), lambsquarter (*Chenopodium album*), lettuce (black-seeded Simpson) and alfalfa was tested. The effect on hypocotyl growth of lettuce and green foxtail was measured. The lengths of roots and hypocotyls were measured to the nearest millimeter.

*Lettuce Seed Germination Bioassay.* Fifty lettuce seeds were placed in Petri dishes containing 40 ml of 0.5% agar and 20, 40, 80, 100, 200, or 500 ppm NDGA. The NDGA was dissolved in methanol as acetone was found to inhibit lettuce seed germination. Seeds were incubated for 24 hr in a growth chamber with 58°, 8-hr nights, and 68°C, 16-hr days. Germinated seeds were counted. Two controls were included: one contained only agar, the second contained agar plus 150 μl of methanol, an amount greater than the maximum amount used to dissolve the NDGA.

*Data Analyses.* The results of the seedling growth bioassays were analyzed separately using the Washington, D.C., Computing Center facilities and Statistical Analysis System (SAS Institute, Inc., Cary, North Carolina, 1982). An initial regression plot of log mean vs. log standard deviation for each experiment was obtained to determine the best transformation of the data (Box et al., 1978). The appropriately transformed data were then subjected to Cochran's test for homogeneity of variances of all treatments and the Duncan multiple-range test for differences between all treatment means.

## RESULTS AND DISCUSSION

*Root Growth Assays.* NDGA brought about dramatic reduction in seedling root growth of all eight of the crop and weed plants tested (Table 1). The mean root length of lettuce, alfalfa, lambsquarter, and barnyard grass treated with 5 ppm of NDGA was less than the respective controls, but the reduction was not statistically significant. The mean root length of all eight of the test plants treated with 10 ppm NDGA was less than the respective controls, and this difference was statistically significant. In fact, with the exception of alfalfa, all test plants had mean root lengths at 10 ppm of roughly half (34–61%) of the mean control root lengths. This reduction by half was achieved at the 20-ppm dose level for alfalfa. It is somewhat surprising that at the 5-ppm dose level statistically significant root growth inhibition was not observed for lettuce, lambsquarter, or barnyard grass and no root growth inhibition was observed for red millet, annual ryegrass, or green foxtail, while each of these plants showed almost 50% reduction of root

TABLE 1. EFFECT OF NDGA ON ROOT GROWTH OF WEED AND CROP SEEDLINGS

| Seedling | NDGA concentration (ppm) | Mean root length (mm)[a] | | Incubation time (hr) | $N^b$ |
|---|---|---|---|---|---|
| Lettuce | 0 | 37.7 | a | 74 | 30 |
| | 5 | 34.9 | a | 74 | 15 |
| | 10 | 19.4 | b | 74 | 15 |
| | 20 | 9.5 | c | 74 | 15 |
| | 40 | 5.1 | d | 74 | 15 |
| | 80 | 2.8 | e | 74 | 15 |
| Alfalfa | 0 | 27.2 | a | 45 | 15 |
| | 0[c] | 25.8 | a, b | 45 | 15 |
| | 5 | 22.5 | b, c | 45 | 15 |
| | 10 | 19.6 | c | 45 | 15 |
| | 20 | 15.4 | d | 45 | 15 |
| | 40 | 8.8 | e | 45 | 14 |
| | 80 | 6.2 | f | 45 | 15 |
| Red millet | 0 | 22.7 | b | 64 | 13 |
| | 0[c] | 27.5 | a, b | 64 | 15 |
| | 5 | 30.0 | a | 64 | 15 |
| | 10 | 16.3 | c | 64 | 15 |
| | 20 | 12.5 | d | 64 | 15 |
| | 40 | 3.9 | e | 64 | 15 |
| | 80 | 1.9 | f | 64 | 15 |
| Annual ryegrass | 0 | 21.8 | a | 48 | 12 |
| | 0[c] | 16.2 | b | 48 | 15 |
| | 5 | 16.2 | b | 48 | 14 |
| | 10 | 9.1 | c | 48 | 14 |
| | 20 | 4.3 | d | 48 | 15 |
| | 40 | 3.9 | d | 48 | 14 |
| | 80 | 4.0 | d | 48 | 15 |
| Perennial ryegrass | 0 | 20.6 | a | 48 | 15 |
| | 10 | 8.1 | b | 48 | 14 |
| | 20 | 4.1 | c | 48 | 15 |
| | 40 | 3.8 | c | 48 | 12 |
| | 80 | 4.0 | c | 48 | 15 |
| Green foxtail | 0 | 22.4 | a | 48 | 20 |
| | 5 | 23.8 | a | 48 | 10 |
| | 10 | 14.1 | b | 48 | 10 |
| | 20 | 3.1 | c | 48 | 10 |
| | 40 | 3.6 | c | 48 | 10 |
| | 80 | 2.8 | c | 48 | 10 |
| Lambsquarter | 0 | 17.0 | a | 48 | 21 |
| | 5 | 16.6 | a | 48 | 12 |
| | 10 | 9.1 | b | 48 | 9 |
| | 20 | 2.5 | c | 48 | 12 |
| | 40 | 2.3 | c | 48 | 12 |
| | 80 | 2.0 | c | 48 | 12 |

TABLE 1. Continued

| Seedling | NDGA concentration (ppm) | Mean root length (mm)[a] | | Incubation time (hr) | $N^b$ |
|---|---|---|---|---|---|
| Barnyard grass | 0 | 22.2 | a | 50 | 23 |
| | 5 | 21.1 | a | 50 | 12 |
| | 10 | 14.7 | b | 50 | 12 |
| | 20 | 6.4 | c | 50 | 12 |
| | 40 | 3.4 | d | 50 | 12 |
| | 80 | 2.7 | d | 50 | 12 |

[a]Means associated with a given test seedling with different letters are significantly different at the $\alpha = 0.05$ level according to Duncan's multiple-range test performed on transformed values. Original values are given. If means for the two controls did not differ significantly, only the combined average is given.
[b]Number of roots measured.
[c]Control with 50 μl of acetone added.

growth at the 10-ppm test level. This may be due to the natural variability of seedling growth, the small sample size measured, or to the necessity of a minimum amount of NDGA being present to bring about measurable inhibition. Once that minimum phytotoxic level is reached, the inhibition is dramatic.

At 20 ppm NDGA, maximum root growth inhibition was observed for annual ryegrass, perennial ryegrass, and green foxtail. Maximum inhibition was observed at 40 ppm for barnyard grass. Lettuce, red millet, and lambsquarter showed further root growth inhibition at 80 ppm NDGA, but this is likely close to the maximum inhibition level for them as their mean root lengths at the 80 ppm level approached the length of the untreated newly germinated seeds. Alfalfa, with a mean root length of 6.2 mm at 80 ppm NDGA appears to be less sensitive to NDGA than are the other seven plants.

*Hypocotyl Length Assays.* NDGA inhibited the hypocotyl growth of both lettuce and green foxtail seedlings (Table 2). Five ppm of NDGA brought about significant hypocotyl growth inhibition in lettuce; at 10 ppm hypocotyl growth was reduced by 50% and maximum inhibition was reached. Thus, although lettuce hypocotyl growth appears more sensitive to NDGA concentration than does lettuce root growth, the maximum growth inhibition was less for hypocotyl growth. For green foxtail only an 80-ppm level of NDGA gave hypocotyl growth inhibition statistically different than the control, and hypocotyl growth was reduced by only 37% at this level.

*Lettuce Seed Germination Assays.* NDGA did not inhibit the germination of lettuce seeds up to a dose level of 500 ppm (Table 3). At 500 ppm, NDGA was soluble in the warm 0.5% agar test solution, but precipitated

TABLE 2. EFFECT OF NDGA ON HYPOCOTYL LENGTHS OF LETTUCE AND GREEN FOXTAIL

| Seedling | NDGA concentration (ppm) | Mean hypocotyl length (mm)[a] | | $N$[b] |
|---|---|---|---|---|
| Lettuce | 0 | 12.1 | a | 30 |
| | 5 | 7.3 | b | 15 |
| | 10 | 5.5 | c | 15 |
| | 20 | 6.0 | c | 15 |
| | 40 | 5.9 | c | 15 |
| | 80 | 5.5 | c | 15 |
| Green foxtail | 0 | 11.9 | a | 20 |
| | 5 | 10.8 | a | 10 |
| | 10 | 11.0 | a | 10 |
| | 20 | 9.7 | a, b | 10 |
| | 40 | 10.0 | a, b | 10 |
| | 80 | 7.5 | b | 10 |

[a]See footnote *a*, Table 1.
[b]Number of seedlings measured.

TABLE 3. EFFECT OF NDGA ON LETTUCE SEED GERMINATION

| NDGA concentration (ppm) | Germination (%) | Number of seeds |
|---|---|---|
| 0 | 93 | 100 |
| 0[a] | 96 | 50 |
| 20 | 94 | 50 |
| 40 | 94 | 50 |
| 80 | 100 | 50 |
| 100 | 100 | 50 |
| 200 | 94 | 51 |
| 500[b] | 98 | 50 |

[a]Control with 150 $\mu$l methanol added.
[b]Some NDGA had precipitated upon standing.

upon standing in the growth chamber. At this upper solubility level of NDGA in the test system, no inhibition of seed germination was observed.

## CONCLUSIONS

NDGA does not inhibit lettuce seed germination, but does inhibit root growth and, to a lesser extent, hypocotyl growth. At the higher test concentrations, plant roots were shrunken and brown, an indication of the phyto-

toxic nature of NDGA (Rice, 1979). NDGA almost certainly contributes to the observed allelopathic nature of the creosote bush. Since NDGA is present in such high concentrations in the leaves of creosote bush, it may be the major contributor of the observed allelopathic properties.

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# EFFECT OF SOME FLORAL SCENTS ON HOST FINDING BY THRIPS (INSECTA: THYSANOPTERA)

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**Abstract**—The role of floral scents in host location by flower-dwelling thrips is investigated by experiment in the field. The scent of anisaldehyde significantly increased the catches of seven species of flower-dwelling thripid, but had no significant effect on three species of cereal thripid and one species of flower-dwelling aeolothripid. The catches of white (without UV) traps were increased by a factor of 3.3 to 8.3 in the presence of the scent.

**Key Words**—Thysanoptera, thrips, attractant, scent traps, color traps, flowers, cereals, anisaldehyde, myrcene, eugenol, geraniol.

## INTRODUCTION

There is evidence that the primitive angiosperms were not exclusively beetle pollinated and that some were closely associated with, and probably pollinated by, Thysanoptera (Gottsberger, 1974, 1977; Thien, 1980). Odor is probably an old "secondary attractant" (Faegri and van der Pijl, 1979), so that thrips may have been associated with scented flowers throughout the evolution of the angiosperms.

It is likely that flower-dwelling thrips are aided in finding flowers while in flight by the scent cues that have evolved in response to legitimate insect pollinators. Annand (1926) and Appanah and Chan (1981) observed that plants with perfume seem to have more thrips on them than those without, which appears to support this hypothesis. In this paper, the effect of flower scents on host location by thrips is investigated and discussed.

I have tested four scents in association with white (without UV) water traps. This color has already been shown to catch more flower thrips than several other colors (Walker, 1974; Kirk, 1984a,b), so the combined influ-



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ence of color and scent can be assessed. One of the compounds (anisaldehyde) was tested further.

The four scents represent chemical groups commonly found in floral fragrances: open-chain monoterpenes (geraniol, myrcene) and simple aromatics (eugenol, anisaldehyde) (Williams, 1983), and have all been identified in essential oils of many flowers (Gildemeister and Hoffmann, 1928; de Naves and Mazuyer, 1947; Attaway et al., 1966; Loper, 1972). Anisaldehyde is not only present in flower scents (de Naves and Mazuyer, 1947; Wakayama et al., 1971; Nilsson, 1979; Lindeman et al., 1982), but is a metabolic product of some wood-rotting fungi and can be the chief contributor to the characteristic odor (Birkinshaw et al., 1952).

The influence of scent on flying thrips has been demonstrated in the field several times. In India, Howlett (1914) caught many thrips in water traps scented with benzaldehyde, anisaldehyde (4-methoxybenzaldehyde), salicaldehyde (2-hydroxybenzaldehyde), or cinnamaldehyde (3-phenyl-2-propenal). Morgan and Crumb (1928) in the United States list several chemicals that caught more thrips than unscented controls. All of the above aromatic aldehydes appear at or near the top of their list. Uchida (1973) patented the use of anisaldehyde and (or) cinnamaldehyde in traps for thrips, after using such traps successfully. In New Zealand, Penman et al. (1982) found that sticky traps scented with ethyl nicotinate caught many more *Thrips obscuratus* (Crawford) than the controls. Evans (1932), however, obtained no positive results for *Thrips imaginis* Bagnall caught in traps scented with three of the above aldehydes, citral, or geraniol.

Some experiments with olfactometers have demonstrated an influence of scent on walking thrips (Holtmann, 1963; Syed, 1978), but these results cannot necessarily be extrapolated to flying insects in the field (Snapp and Swingle, 1929).

## METHODS AND MATERIALS

Water traps (painted plastic dishes of diameter 165 mm and depth 60 mm) were filled with tap water to 20 mm below the rim, and 0.3 ml of Teepol detergent was added to each. They were placed on level grassy surfaces, so that the tops were about 90 mm above ground level. All the traps were matt black on the outside and were painted white without UV (titanium dioxide pigment) inside. The diffuse reflectance spectrum of the white paint is shown in Figure 1A of Kirk (1984a). All the experiments were run during periods of relatively low wind speeds.

Scent was released from glass specimen tubes (length 50 mm, diameter 10 mm) with a dental roll wick, cut to a length of 20 mm, projecting 10 mm above the top. A strip of filter paper ran the length of the tube and acted as a

wick between the dental roll and the chemical. The chemical and wick were replaced at least every 48 hr. The following undiluted chemicals were used as scents: anisaldehyde (4-methoxybenzaldehyde) supplied by BDH Chemicals Ltd.; beta-myrcene (7-methyl-3-methylene-1,6-octadiene), and eugenol [2-methoxy-4-(2-propenyl) phenol] supplied by Sigma Chemical Company; and geraniol [(*E*)-3,7-dimethyl-2,6-octadien-1-ol] supplied by Koch-Light Laboratories Ltd. Tap water was the control.

The insects caught in the traps were filtered out and stored in 70% alcohol. Thrips were identified under a stereoscopic microscope or mounted in polyvinyl lactophenol and examined under a compound microscope.

A $\log_{10} (n + 1)$ transformation was used in the analysis of the results, because the effects were multiplicative, and to homogenize the variance.

*Scent Traps at Taunton.* Traps were left in a meadow (map reference ST 177258) near Taunton, England, from 1000 to 1800 Greenwich mean time on August 25, 1982. There were two replicates of each of four scents (anisaldehyde, myrcene, eugenol, and geraniol) and four replicates of the control. One scent dispenser was taped to the outer edge of each trap. The traps were placed at intervals of 10 m around the perimeter of a circle (diameter 38.2 m) so that each trap was opposite a trap with the same scent. The four controls were at intervals of 90° around the circle. Because of the many treatments and low level of replication, this circular design with similar treatments opposite each other was used to reduce effects of site inhomogeneity, directional effects, or interference between treatments. All thrips were identified to species. The data for all species were combined, and the mean trap catches for the treatments and control were compared using analysis of variance and Dunnett's $t$ test.

*Scent Traps at Cambridge.* Traps were left out each day from August 14 to 17, 1983 in the middle of a sports field (map reference TL 430574) in Cambridge, England. There were six replicates of a scent (anisaldehyde), and six replicates of the control. One scent dispenser was held in the middle of each trap by a wire. The two types of trap were arranged at alternate corners of a 10-m-square grid that covered an area of 20 m × 30 m. The catches for each trap were combined over the four days. Results for each species were analyzed separately and analysis of variance was used to test for scent–sex interactions. The mean trap catches of the treatment and control were compared using $t$ tests.

## RESULTS

*Scent Traps at Taunton.* In this pilot study, four scents were tested in order to select one of them for further tests. Eleven species of thripid were caught. When the total numbers of thrips of all species were analyzed, there

were significant overall differences between the four scents and control ($F_{4,7} = 20.7$; $P < 0.001$). Comparison of each of the scents with the control showed that myrcene caught significantly fewer and anisaldehyde caught significantly more (Table 1). Eugenol and geraniol were not significantly different. A breakdown of the results for the four commonest species (*Thrips major* Uzel, *Thrips flavus* Schrank, *Frankliniella intonsa* (Trybom), and *Thrips pillichi* Priesner) in Table 1 shows a similar pattern for each. All these species are flower thrips (Mound et al., 1976). Although eugenol and geraniol were not significantly different from the control, further experiments may well reveal an effect, particularly if samples are large enough to consider species separately. For example, geraniol appears to have been catching *Thrips flavus* in particularly large numbers.

Anisaldehyde increased the catch of thrips by a factor of more than four, even though a color of trap was used that can increase the catch by a factor of a hundred over other colors (Kirk, 1984a). This scent was therefore tested further.

*Scent Traps at Cambridge.* Seventeen species of thrips were caught over the four days. Nine species (*Limothrips cerealium* Haliday, *Limothrips denticornis* Haliday, *Frankliniella tenuicornis* (Uzel), *Aeolothrips intermedius* Bagnall, *Thrips vulgatissimus* Haliday, *Thrips tabaci* Lindeman, *Thrips major*, *Thrips pillichi*, and *Frankliniella intonsa*) were in sufficiently large numbers for statistical analysis. Only females were caught of *L. cerealium*, *L. denticornis*, and *T. vulgatissimus*. Enough males of *A. intermedius*, *F.*

TABLE 1. SCENT TRAP CATCHES AT TAUNTON IN 1982 FOR FOUR SCENTS AND A CONTROL.

| Species | Trap scent | | | | |
|---|---|---|---|---|---|
| | Water (control) | Myrcene | Eugenol | Geraniol | Anisaldehyde |
| Transformed data[a] | | | | | |
| All thrips | 1.23 | 0.90* | 1.28[NS] | 1.39[NS] | 1.85** |
| Untransformed data[b] | | | | | |
| All thrips | 16.2 | 7.0 | 18.5 | 24.0 | 72.0 |
| *Thrips major* | 3.5 | 1.0 | 3.5 | 1.5 | 31.5 |
| *Thrips flavus* | 3.8 | 0.0 | 8.5 | 11.5 | 11.0 |
| *Frankliniella intonsa* | 4.2 | 2.5 | 2.0 | 4.5 | 12.0 |
| *Thrips pillichi* | 2.8 | 3.0 | 1.5 | 3.0 | 12.0 |

[a]Means of $\log_{10}(n + 1)$ transformed data for all thrips combined. The statistical significance of the differences between treatments and control, using Dunnett's $t$ test, is shown (NS not significant; $*P < 0.05$; $**P < 0.01$). The standard error of the difference was 0.093, with seven degrees of freedom.

[b]Means per traps for all thrips species combined, and four of the most common species of thrips.

*tenuicornis*, and *T. pillichi* were present to test for scent–sex interactions. These were not significant at the 5% level. Inspection of the data gave no suggestion of an interaction for the other species either, *F. intonsa*, *T. tabaci*, and *T. major*, so the sexes were combined in all cases.

The effect of scent was not significant for species 1–4 (Table 2), but was significant for species 5–9 ($P < 0.001$ in all cases). Anisaldehyde caught between 3.3 and 8.3 times more individuals than the control for each of these five species. Since the relative effect of trap color has been measured for most of these species (Kirk, 1984a), a rough estimate of the combined influence of scent and color can be produced. For example, 33 times more *Thrips major* were caught by white without UV than by green (Table 1 of Kirk, 1984a), and the scent of anisaldehyde increased the catch in white without UV traps by a factor of 5.1. This gives an estimated 170 times more *Thrips major* landing on a white without UV surface scented with anisaldehyde than on an unscented green surface. Similarly, an estimated 255 times more would land on this scented white surface than on an unscented black surface. The

TABLE 2. SCENT TRAP CATCHES OF THRIPS AT CAMBRIDGE IN 1983 FOR ANISALDEHYDE AND A CONTROL[a]

| Species | Trap scents: Water (control) | Trap scents: Anisaldehyde | Ratio of scent to control | SED | $P$ |
|---|---|---|---|---|---|
| 1. *Limothrips* | 82 | 67 | 0.8 | | |
| *cerealium* | 1.16 | 1.07 | | 0.06 | 0.20 |
| 2. *Limothrips* | 120 | 167 | 1.4 | | |
| *denticornis* | 1.27 | 1.43 | | 0.12 | 0.23 |
| 3. *Frankliniella* | 129 | 124 | 1.0 | | |
| *tenuicornis* | 1.32 | 1.30 | | 0.12 | 0.91 |
| 4. *Aeolothrips* | 37 | 31 | 0.8 | | |
| *intermedius* | 0.72 | 0.77 | | 0.19 | 0.78 |
| 5. *Thrips* | 24 | 200 | 8.3 | | |
| *vulgatissimus* | 0.64 | 1.52 | | 0.11 | $<0.001$ |
| 6. *Thrips tabaci* | 84 | 308 | 3.7 | | |
| | 1.16 | 1.71 | | 0.07 | $<0.001$ |
| 7. *Thrips major* | 33 | 169 | 5.1 | | |
| | 0.79 | 1.46 | | 0.07 | $<0.001$ |
| 8. *Thrips pillichi* | 78 | 259 | 3.3 | | |
| | 1.13 | 1.64 | | 0.07 | $<0.001$ |
| 9. *Frankliniella* | 14 | 71 | 5.1 | | |
| *intonsa* | 0.44 | 1.09 | | 0.14 | $<0.001$ |

[a]For each species, the first line gives the total catch for each scent and the ratio of these catches. The second line gives means of the $\log_{10}(n + 1)$ transformed data for each scent, their standard errors of the difference (SED), and the significance level, using a $t$ test (10 degrees of freedom).

traps were distinguished while 10 m apart, so scent is probably influencing thrips at distances of less than 10 m from the source.

Species 1–3 are grass-feeders, while species 4–9 are each associated with a wide range of species of flower (Mound et al., 1976). The scent response follows this ecological division, except for species 4, *Aeolothrips intermedius*. All the species are thripids, apart from 4, which is a predatory aeolothripid (Bournier et al., 1978).

## DISCUSSION

In my earlier experiments with colored traps (Kirk, 1984a), all the flower thrips responded strongly to certain colors, while the cereal thrips responded little or not at all. A similar result was found here with anisaldehyde. Five species of flower thrips responded to the scent, while the same three species of cereal thrips, together with *Aeolothrips intermedius* (a predatory flower thrips), showed no response to the scent. Two species in the genus *Frankliniella* Karny were caught: *F. tenuicornis* is a cereal thrips and did not respond to the scent, while *F. intonsa* is a flower thrips and responded. This supports the hypothesis that there is a relationship between the type of host and the response to anisaldehyde. *Aeolothrips intermedius*, the only aeolothripid caught, responded to colors in the same way as other flower thrips (Kirk, 1984a), but its scent response differed from that of other flower thrips. This may be a general difference between thripids and aeolothripids and could be tested by using scent traps for other common aeolothripid flower thrips, such as *Aeolothrips tenuicornis* Bagnall or *Melanthrips fuscus* (Sulzer).

All the flower thrips caught in the traps are generalists, found in a wide range of flowers (Mound et al., 1976). Generalist thrips might respond to a wide range of scents usually associated with flowers, so that anisaldehyde may be just one of many compounds that stimulate the same response. The efficacy of anisaldehyde in traps is not restricted to flower thrips. It has increased trap catches of a wide range of insects in the field: flies (Morgan and Crumb, 1928); moths (Frost, 1936; Barnes et al., 1954); beetles (Morgan and Crumb, 1928; Uchida, 1973); and solitary bees (Morgan and Crumb, 1928).

No specialist flower thrips were caught during the scent trap experiments. If these species have a scent response, it is likely to be to only a few compounds and thus harder to discover.

The tubes of undiluted scent compound probably produced a higher concentration at the scource than is found in flowers. Many chemicals are known to increase catches at low concentrations and decrease them at high concentrations (Snapp and Swingle, 1929; Perry and Fay, 1967; Rodrigues, 1980). Myrcene reduced the catch of thrips at the concentration used in this experiment, but it might increase catches at lower concentrations. Without

knowing the actual vapor concentration distribution around the flower, it is not easy to relate the concentrations of pure compounds used in experiments to those that occur naturally.

The above experiments do not distinguish among the different mechanisms by which the thrips could have responded to the scent, but they do show that the response occurs in flight before landing. Insect responses in flight to the scent of host plants and flowers are often anemotactic (Hawkes et al., 1978; Brantjes, 1981) and so would be impossible in completely still air. Pheromone responses are also commonly anemotactic (David et al., 1982). If the responses of thrips were the same, they could only be effective within a very narrow range of wind speeds. It seems probable that thrips could use a scent cue more efficiently as an arrestant, or to stimulate a visual response, than for anemotaxis, because the cue could then also be used when the air is completely still. Scent responses that stimulate a visual response and do not involve anemotaxis are known in several types of flower-visiting insects (Kugler, 1956; Manning, 1957).

Research on the influence of scent under natural conditions is handicapped by the shortage of positive identifications of floral fragrances (Williams, 1983) and the technical problems of measuring their concentrations. Valuable information could be gained from experiments on specialist flower thrips when the host volatiles are known. Such studies could also use the naturally occurring scent mixtures, which do not necessarily give the combined effects of the components (e.g., Langford et al., 1943; Ackerman, 1983). When the mechanism of the scent response by thrips is known, it may be possible to exploit it to prevent infestation or spread of infestation. High concentrations of some volatiles may repel thrips and so could be used to protect horticultural blooms. Flowers that are regularly damaged by flower thrips, either visibly or through removal of pollen (Kirk, 1984c), might be less damaged if they were a different color or had no scent or a different scent. Knowledge of the scents and colors involved could be of use to plant breeders aiming to develop cultivars that are less liable to thrips attack.

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# RHODANESE IN INSECTS

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**Abstract**—Forty-four species of insects were assayed for the presence of rhodanese, an enzyme generally considered to be responsible for the detoxification of cyanide. Rhodanese was found to be widely distributed in both adults and larvae and was not restricted to those species which encounter exogenous cyanide through feeding on cyanogenic plants. These results indicate that cyanide detoxification is unlikely to be the primary role for rhodanese in insects.

**Key Words**—Cyanide, rhodanese, cyanogenesis, detoxification, *Lotus corniculatus, Vicia sativa, Zygaena,* Insecta, chalk grassland.

## INTRODUCTION

The ability of the enzyme rhodanese to detoxify cyanide has been known for many years (Lang, 1933). Rhodanese has a variety of other functions, however (reviewed by Westley, 1973), and Volini and Alexander (1981) have suggested that the traditional role of rhodanese as a sulfur donor to cyanide may merely be complementary to its routine function as a sulfur donor to proteins.

Insects, in general, are much less sensitive to cyanide than mammals, with certain groups being particularly resistant (Povolny and Weyda, 1981; Bernays, 1982; Brattsten et al., 1983). It has generally been assumed that this insensitivity is dependent upon rhodanese (e.g., Dowd et al., 1983), but there is evidence that alternative detoxification systems may also be important. Parsons and Rothschild (1964), for example, showed that the larvae and adults of *Malacosoma neustria* L. contained only trace quantities of rhodanese, and yet this species had the ability to feed on the highly cyanogenic leaves of laurel (*Prunus laurocerasus* L.). The larvae of *Zygaena* species are themselves cyanogenic and are extremely resistant to cyanide, yet contain little, if any, rhodanese (Jones et al., 1962). The source of this resistance may be a combina-



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tion of an insensitive cytochrome oxidase and the action of another cyanide detoxifying enzyme, β-cyanoalanine synthetase, the presence of which has been detected in *Zygaena* (R. Davis, personal communication). Long and Brattsten (1982) and Brattsten et al. (1983) have shown that the larvae of *Spodoptera eridania* Cramer can acquire a tolerance of cyanide and that the mechanism of this resistance is not based on rhodanese.

The ability of animals to feed on cyanogenic plants should be correlated with the amounts of detoxifying enzymes that they possess (Conn, 1979). In this paper we describe the results of a survey for the presence of rhodanese amongst a wide range of insects. These include species known to feed on *Lotus corniculatus* L. and *Vicia sativa* L., plants which possess cyanogenic leaves, petals and cyanogenic seeds, respectively.

## METHODS AND MATERIALS

The insects studied are listed in Table 1. Most of them were collected from an area of chalk grassland at Wharram Quarry in North Yorkshire. Additional material was obtained from the following East Yorkshire locali-

TABLE 1. VARIATION IN LEVELS OF RHODANESE RECORDED IN INSECTS

| Species | Stage[a] | No. of replicates | Rhodanese (units/g fresh wt)[b] | |
|---|---|---|---|---|
| | | | Range | Mean |
| Orthoptera | | | | |
| *Omocestus viridulus* (L.) | A | 3 | ND[c]–<0.01 | <0.01 |
| *Myrmeleotettix maculatus* (Thun.) | A | 16 | 0.01–0.05 | 0.02 |
| *Chorthippus brunneus* (Thun.) | A | 20 | 0.01–0.05 | 0.03 |
| Dermaptera | | | | |
| *Forficula auricularia* L. | A | 3 | 0.05–0.05 | 0.05 |
| Heteroptera | | | | |
| *Myrmus miriformis* (Fallen) | A | 3 | 0.16–0.27 | 0.23 |
| *Plagiognathus chrysanthemi* (Wolff)[d] | A | 3 | 1.68–2.38 | 2.03 |
| *Orthotylus* sp. | A | 3 | 2.04–2.84 | 2.35 |
| Homoptera | | | | |
| *Philaenus spumarius* (L.)[d] | A | 3 | 0.14–0.48 | 0.34 |
| *Neophilaenus lineatus* (L.) | A | 5 | 0.18–0.77 | 0.49 |
| *Megopthalmus* sp. | N | 5 | 0.05–0.23 | 0.19 |
| *Megopthalmus* sp. | A | 4 | 0.20–0.23 | 0.22 |
| *Agallia brachyptera* (Boheman) | A | 3 | 1.45–2.66 | 1.98 |
| *Agallia venosa* (Fallen) | A | 2 | 0.61–0.95 | 0.78 |
| *Aphrodes bicinctus* (Schrank)[d] | N | 3 | 0.11–0.11 | 0.11 |
| *Turrutulus socialis* (Flor) | A | 3 | 0.89–1.38 | 1.07 |
| *Arthaldens pascuellus* (Fallen) | A | 3 | 3.20–6.20 | 4.35 |

TABLE 1. Continued

| Species | Stage[a] | No. of replicates | Rhodanese (units/g fresh wt)[b] | |
|---|---|---|---|---|
| | | | Range | Mean |
| *Paluda adumbrata* Sahlberg. | A | 3 | 0.27–0.95 | 0.67 |
| *Eupteryx notata* Curtis | A | 4 | 1.45–4.38 | 2.64 |
| *Aphis loti* Kaltenbach[d] | A | 3 | 0.73–0.93 | 0.84 |
| *Acyrthosiphon loti* (Theobald)[d] | A | 3 | N.D.–0.09 | 0.03 |
| Coleoptera | | | | |
| *Coccinella 7-punctata* L. | A | 2 | 0.05–0.07 | 0.06 |
| *Coccinella 11-punctata* L. | A | 3 | 0.07–0.14 | 0.11 |
| *Meligethes aeneus* (Fabr.) | A | 3 | 0.41–0.59 | 0.51 |
| *Bruchus atomarius* (L.)[e] | L | 6 | 0.09–0.41 | 0.20 |
| *Bruchus atomarius* (L.)[e] | A | 3 | ND | |
| *Bruchus loti* Paykull | A | 4 | 0.07–0.57 | 0.30 |
| *Crepidodera ferruginea* (Scop.) | A | 3 | 0.23–0.54 | 0.33 |
| *Aphthona atrovirens* Foerst. | A | 2 | 0.43–1.63 | 1.03 |
| *Altica* sp. | A | 3 | 0.05–0.18 | 0.11 |
| *Apion loti* Kirby[d] | L | 3 | 0.27–1.45 | 0.84 |
| *Apion loti* Kirby[d] | A | 8 | 1.63–4.63 | 3.37 |
| *Apion nigritarse* Kirby | A | 3 | 0.77–1.79 | 1.40 |
| *Apion assimile* Kirby | A | 5 | 0.77–5.11 | 2.57 |
| *Phyllobius roboretanus* Gredler | A | 7 | 0.14–0.64 | 0.28 |
| *Sitona striatellus* Gyllenhal | A | 3 | 0.02–0.18 | 0.09 |
| *Hypera plantaginis* (Degeer)[d] | L | 3 | 0.07–0.45 | 0.29 |
| *Hypera plantaginis* (Degeer)[d] | A | 3 | 0.45–0.61 | 0.51 |
| Lepidoptera | | | | |
| *Micropterix aruncella* (Scop.) | A | 5 | 1.18–4.06 | 2.43 |
| *Zygaena filipendulae* (L.)[d] | L | 3 | ND | |
| *Zygaena filipendulae* (L.)[d] | A | 3 | <0.01–0.01 | <0.01 |
| *Cydia nigricana* (Fabr.)[e] | L | 7 | 0.84–2.07 | 1.30 |
| *Erynnis tages* (L.)[d] | L | 1 | 0.02 | |
| *Polyommatus icarus* (Rott.)[d] | L | 4 | 0.09–0.16 | 0.13 |
| *Polyommatus icarus* (Rott.)[d] | A | 1 | 0.11 | |
| *Coenonympha pamphilus* (L.) | A | 3 | ND–0.02 | <0.01 |
| *Tyria jacobaeae* (L.) | L | 1 | 0.01 | |
| Diptera | | | | |
| *Contarinia loti* (Degeer)[d] | L | 6 | 0.59–1.62 | 0.88 |
| Hymenoptera | | | | |
| *Tenthredo acerrima* Benson[d] | L | 3 | <0.01–0.05 | |
| *Eurytoma platyptea* (Walker)[d] | A | 1 | 2.20 | |
| *Entedon diotimus* (Walker) | A | 1 | ND | |

[a]Nymph/larva or adult.
[b]As defined by Sorbo (1955).
[c]None detected.
[d]Species known to feed regularly on *Lotus corniculatus*.
[e]Species known to feed regularly on *Vicia sativa*.

ties: Hull (*Chorthippus brunneus*), Hessle (*Forficula auricularia*), Cottingham (*Aphis loti* and *Acyrthosiphon loti*), and Gilberdyke (*Coccinella 7-punctata* and *C. 11-punctata*). The specimens of *Bruchus atomarius* were obtained from Donnington Castle, Newbury, Berkshire, and the *Cydia nigricana* were from Raglan, Gwent.

The rhodanese content of the insects was assayed using the method of Sorbo (1955) modified for small volumes of homogenate. Insects were stored frozen for periods of up to one month before analysis. They were homogenized by an Araldite motor-driven pestle in 100–200 $\mu$l of chilled deionized water contained in a 1-ml polypropylene microcentrifuge tube. The crude homogenate was spun down for 10 min in a microcentrifuge and the supernatant assayed at room temperature ($\pm 21°C$) for 10 min. Formaldehyde was used to terminate the reaction (Miller and Conn, 1980). The assay mixture was then centrifuged at 21,000 rpm for 30 min at 2°C and the supernatant read immediately at 460 nm in a 0.1-ml cuvette. Individual insects ranged in weight from 0.001 to 0.1 g and for most of the species it was necessary to combine several specimens within each replicate.

## RESULTS

The results (Table 1) show that rhodanese is present in detectable quantities in both the larvae and adults of a wide variety of insects and is not restricted to those species that regularly feed on cyanogenic plants. Between-species comparisons of rhodanese levels can only be tentative because there is generally considerable variation in the activity recorded for replicates of the same species and because fresh weight comparisons are liable to be biased by variation in the water content of different species and different stages of the same species. Nonetheless, these results do suggest that the rhodanese levels of species which feed on cyanogenic plants are not appreciably greater than those of other species.

## DISCUSSION

The rhodanese content of *Hypera plantaginis* and *Polyommatus icarus* has been assayed previously by Parsons and Rothschild (1964). The levels of rhodanese activity they recorded are considerably higher than those detected during the present study. In an earlier study, Jones et al. (1962) found that rhodanese was absent in *Zygaena filipendulae*. We have also failed to detect rhodanese activity in the larvae of this species, but did record very low levels in the adult. *Zygaena* spp. are themselves cyanogenic and synthesize their cyanogenic glucosides de novo (Jones et al., 1962; Davis and Nahrstedt, 1982). It could be argued that the presence of cyanide detoxifying enzymes would be disadvantageous for insects, like *Zygaena* spp., which employ cyanogenesis as a chemical defence. The presence of $\beta$-cyanoalanine synthetase in *Z. filipen-*

*dulae* (R. Davis, personal communication), however, does not support that argument. As the major food plant of *Z. filipendulae* is itself cyanogenic (Table 1) (Jones et al., 1962), it is perhaps not surprising to find that even these cyanogenic larvae have the capability for detoxifying exogenous HCN.

Rhodanese has been shown to be almost ubiquitous among a taxonomically varied sample of insects. Trace quantities of cyanogenic glucosides may be present in most plants (Jones, 1979), but these would seem unlikely to account for the quantities of rhodanese that were detected, and we conclude that insects may have a general requirement for rhodanese which is independent of their need to detoxify cyanide. These results also bring into question whether rhodanese is employed to detoxify cyanide in those insects which routinely feed on cyanogenic plants. These species do not appear to contain unusually high levels of rhodanese activity, which implies that either the basal rhodanese content of insects is able to cope with a cyanogenic diet or that other detoxifying enzymes are also involved. It is instructive to compare the rhodanese levels of *Cydia nigricana* (Lepidoptera) and *Bruchus atomarius* (Coleoptera), two species which feed on extremely cyanogenic seeds. Moderately high levels of rhodanese activity were recorded from *C. nigricana,* but very little from *B. atomarius* larvae and none from the adults. This may be an example of two species which share the same food item, but have evolved different methods of circumventing its secondary compounds. The $\beta$-cyanoalanine synthetase content of these species is currently being investigated.

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# SYNTHESIS OF KAIROMONAL 2-ACYLCYCLOHEXANE-1,3-DIONE COMPONENTS OF LARVAL MANDIBULAR GLANDS OF *Ephestia kuehniella*

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**Abstract**—Three components of the larval mandibular glands of *Ephestia (= Anagasta) kuehniella,* 2-(*Z,E*)-hexadeca-12′,14′-dienoylcyclohexane-1,3-dione, 2-oleoylcyclohexane-1,3-dione, and 2-linoleoylcyclohexane-1,3-dione, have been synthesized and shown to be active as kairomones.

**Key Words**—Kairomones, oviposition, 2-(*Z,E*)-hexadeca-12′, 14′-dienoylcyclohexane-1,3-dione, 2-oleoylcyclohexane-1,3-dione, 2-linoleoylcyclohexane-1,3-dione, *Ephestia kuehniella* Zeller (syn. *Anagasta kuehniella,* Zeller), Lepidoptera, Pyralidae, *Nemeritis canescens* (Grav.) [syn. *Venturia canes ens* (Grav.)] Hymenoptera, Ichneumonidae

## INTRODUCTION

The 2-acylcyclohexane-1,3-diones and their derivatives have a very wide range of important biological properties including insecticidal, fungicidal, and insect-behavior-controlling activities (see Akhrem et al., 1978; Mudd, 1981; Mudd and Corbet, 1982). Sixteen 2-acylcyclohexane-1,3-diones have been found (Mudd, 1983) in the larval mandibular glands of *E. kuehniella* and shown to elicit oviposition movements from the parasite *Nemeritis* (Mudd et al., 1984). A general synthesis of these compounds was required to fully investigate their biological properties and potential role in pest control.

Previous attempts to synthesize these natural products by introducing long unsaturated acyl side chains into cyclohexane-1,3-diones at the 2-position by a variety of methods met with no success or led to isomerization or destruction of double bonds in the acyl side chain (Mudd, 1981). Short acyl side chains can be introduced by *C*-acylation with acid anhydrides using Lewis



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acid catalysts (Rogers and Smith, 1955), but yields are very low except for short, saturated aliphatic ($C_2$–$C_4$) or aromatic acyl side chains. Yields can be improved using $BF_3$ as a catalyst at high temperatures (Mudd, 1981), but this results in isomerization of the side-chain double bonds. We report a short synthesis, which retains the geometry of the double bonds, that has been used to synthesize three kairomonally active components from *E. kuehniella* mandibular glands.

## METHODS AND MATERIALS

Infrared spectra were recorded as liquid films on a Perkin-Elmer Infracord or 257 grating spectrometer. [$^1$H] NMR were recorded in $CDCl_3$ with a Jeol JNM-PMX60 spectrometer and [$^{13}$C]NMR in $CDCl_3$ with a Jeol JNM-PFT-100 spectrometer using $Me_4Si$ as standard ($\delta = 0.00$). High-pressure liquid chromatography (HPLC) was performed using a Gilson HPLC system with a Lichoprep 15-25 silica column (hplc Technology Ltd., 25 cm × 22.5 mm). Thin-layer chromatography was performed with Merck precoated silica plates (25 × 25 cm, 0.25 cm thick) developed with either chloroform or chloroform–0.1% trifluoroacetic acid (TFA) and visualized with iodine vapor.

Electron-impact mass spectra (EIMS) were determined (VG Micromass 70-70F with Data system 2000) as probe tip samples at 70 eV with source temperature 200°C.

*11-Carbomethoxyundecyl Triphenylphosphonium Bromide VII.* Methyl 12-bromododecanoate (VIII, 19.5 g, prepared from 12-bromododecanoic acid (Aldrich), and triphenyl phosphine (19.6g) were refluxed in dry xylene (200 ml) for 48 hr in a 500-ml round-bottom flask (see Figure 1). On cooling, a clear brown gum formed from which the excess xylene was decanted, and the gum was triturated several times with dry benzene to remove unreacted starting material. After evaporation of residual benzene, the phosphonium salt (39 g) (99%) crystallized overnight in the deep freeze.

*Methyl*(Z,E)*-hexadeca-12,14-dienoate V.* Sodium bis(trimethylsilyl)amide (8 g, Fluka AG) in dry benzene (50 ml) was added to the phosphonium salt VII (20g) in dry benzene, and the mixture was stirred vigorously for 3 hr at room temperature to give a deep red solution of the ylide (Bestmann et al., 1976). The mixture was cooled in ice, and freshly distilled crotonaldehyde (3.5 ml) in dry benzene (10 ml) was added dropwise, under dry nitrogen, over a 3-hr period. After stirring overnight at room temperature, the solvent was removed by rotary evaporation, and the viscous residue was stirred with pentane causing precipitation of triphenylphosphine oxide which was removed by filtration. The filtrate was cooled to −20°C causing further precipitation of triphenylphosphine oxide. After filtration and evaporation of the

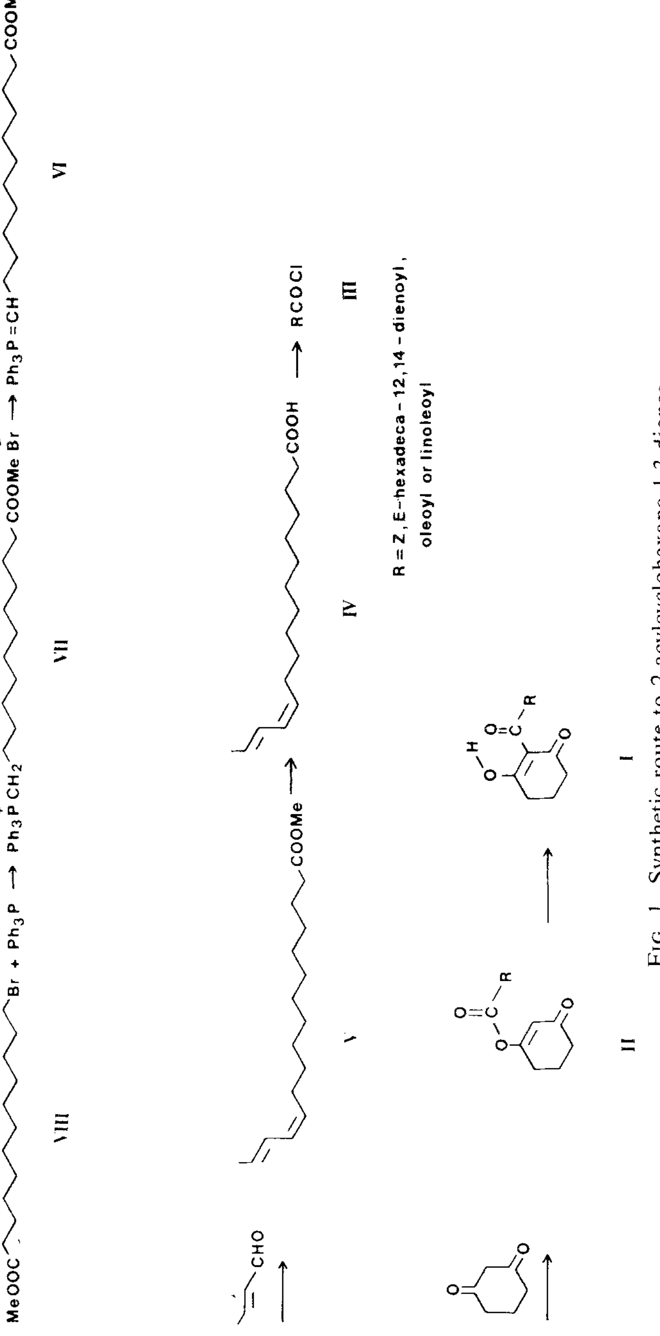


FIG. 1. Synthetic route to 2-acylcyclohexane-1,3-diones.

solvent, the residue (7 g) was purified by HPLC on a silica column with dichloromethane as the eluent using the diene absorption at 233 nm to detect the product. The diene ester (2.5 g) (26% from phosphonium salt) was obtained as a mixture of *Z,E* and *E,E* isomers in the ratio of approximately 90:10 determined by [$^{13}$C]NMR. The *E,E* isomer was removed from the mixture by formation of the Diels-Alder adduct (Nesbitt et al., 1973) with excess tetracyanoethylene, by stirring overnight in tetrahydrofuran at room temperature. The unreacted *Z,E* isomer V was obtained by HPLC as described above. [$^{13}$C]NMR ($CDCl_3$,) 173.8(C=O), 129.5, 128.7(2C), 127.2 (—C=C—C=C—), 51.2 ($CH_3O$—), 34.0 ($CH_2COO$—), 29.8, 29.6, 29.3, 27.7, 25.0, 18.3. *m/z* 266($M^+$, 28) 235(9), 234(6), 192(5), 95(33), 81(80), 68(100).

(Z,E)-*Hexadeca-12,14-dienoic Acid IV.* The ester (V, 790 mg) was added to methanolic KOH (50 ml) in a 100-ml conical flask and stirred overnight at room temperature under nitrogen. The mixture was cooled in ice, carefully acidified with conc. HCl and transferred to a separating funnel where it was extracted with ether (3 × 50 ml). The combined ether extracts were washed with water (3 × 50 ml), brine (50 ml), and dried over magnesium sulfate. After filtration, the solvent was evaporated to give a white solid, mp 42–43° C (580 mg) (78%). [$^{13}$C]NMR ($CDCl_3$,) 180.1 (C=O), 129.8, 128.8, 128.5, 127.1 (—C=C—C=C—), 34.2 ($CH_2COO$—), 29.9, 29.5. *m/z* 252 ($M^+$, 19), 109(13), 96(25), 81(94), 68(100).

From this point all syntheses were similar and the same experimental details apply to all.

*Acid Chloride III.* The acid (0.018 mol) was added to oxalyl chloride (10

TABLE 1. [$^{13}$C] NMR SPECTRA OF ENOL ESTERS(II)

| | | Ring atoms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 1′ | 2′ | 3′ | 4′ |
| II | Cyclohex-2-enone-3-yl oleate | 198.8 | 117.4 | 170.0[a] | 34.5 | 21.4 | 36.8 | 169.7[a] | 36.8 | 24.7 | 29.4 |
| | Cyclohex-2-enone-3-yl linoleate | 199.0 | 117.5 | 170.0[a] | 34.5 | 21.4 | 36.8 | 169.8[a] | 36.8 | 24.7 | 29.4 |
| | Cyclohex-2-enone-3-yl-(*Z, E*)-hexadeca-12′, 14′-dienoate | 199.3 | 117.4 | 170.1[a] | 34.5 | 21.3 | 36.7 | 170.1[a] | 36.7 | 24.7 | 29.5 |
| I | 2-Oleoylcyclohexane-1,30-dione[c] | 198.5 | 113.0 | 195.0 | 33.3 | 19.2 | 38.8 | 206.2 | 40.1 | 24.7 | 29.4 |
| | 2-Linoleolycyclohexane-1,3-dione[c] | 198.5 | 113.0 | 195.0 | 33.4 | 19.3 | 38.9 | 206.2 | 40.1 | 24.7 | 29.4 |
| | 2-(*Z, E*)-Hexadeca-12′,14′-dienoyl-cyclohexane-1,3-dione[c] | 198.5 | 113.0 | 195.1 | 33.3 | 19.1 | 38.8 | 206.2 | 40.1 | 24.7 | 29.5 |

[a,b]Shifts may be transposed.
[c]For consistency within the table, C-3 was assigned to the enolized carbon not C-1 (see Figure 2), so that some shifts of the ring C atoms (C-1 and C-3; C-4 and C-6) are transposed compared with a previous publication (Mudd, 1981).

FIG. 2. Ring atom numbering for compounds in Table 1.

ml) and stirred under nitrogen at room temperature for 1 hr until the evolution of gas subsided. The mixture was then gently refluxed for 30 min under nitrogen and the excess oxalyl chloride was removed by rotary evaporation. After checking by infrared to ensure that the reaction was complete ($\nu_{C=O}$ 1800 $cm^{-1}$ for the acid chloride, and $\nu_{C=O}$ 1700 $cm^{-1}$ for the acid) and impurities (e.g., acid anhydride $\nu_{C=O}$ 1820 and 1760 $cm^{-1}$) were absent, the product was used for the next step without further purification.

*Enol Ester II.* The acid chloride III (0.14 mol) in dry chloroform (30 ml) was added to a solution of cyclohexane-1,3-dione (0.14 mol) in dry pyridine (2 ml), and the solution was stirred for 1 hr at room temperature. The mixture was transferred to a separating funnel with more chloroform and washed with water (40 ml), 0.1 N HCl (2 × 40 ml), water (40 ml), saturated sodium bicarbonate (40 ml), and water (40 ml). The organic layer was dried over magnesium sulfate and evaporated to give the enol ester II as a colorless oil in quantitative yield. [$^{13}$C]NMR (Table 1, Figure 2) and thin-layer chromatog-

AND CORRESPONDING 2-ACYLCYCLOHEXANE-1,3-DIONES(I)

| Side-chain atoms | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5′ | 6′ | 7′ | 8′ | 9′ | 10′ | 11′ | 12′ | 13′ | 14′ | 15′ | 16′ | 17′ | 18′ |
| 29.4 | 29.4 | 29.7 | 27.2 | 129.7 | 130.0 | 27.2 | 29.7 | 29.4 | 29.4 | 29.4 | 32.0 | 22.8 | 14.1 |
| 29.4 | 29.4 | 29.4 | 27.2 | 130.1 | 128.0 | 25.7 | 128.0 | 130.1 | 27.2 | 29.1 | 31.6 | 22.6 | 14.1 |
| 29.5 | 29.5 | 29.5 | 29.5 | 29.5 | 28.4 | 27.7 | 129.7$^b$ | 128.7$^b$ | 128.7$^b$ | 127.2$^b$ | 18.3 | | |
| 29.4 | 29.4 | 29.7 | 27.2 | 129.8 | 129.8 | 27.2 | 29.7 | 29.4 | 29.4 | 29.4 | 32.0 | 22.8 | 14.1 |
| 29.4 | 29.4 | 29.4 | 27.3 | 130.1 | 128.1 | 25.7 | 128.1 | 130.1 | 27.3 | 29.4 | 31.6 | 22.6 | 14.1 |
| 29.5 | 29.5 | 29.5 | 29.5 | 29.5 | 29.5 | 27.7 | 129.8$^b$ | 128.7$^b$ | 128.7$^b$ | 127.1$^b$ | 18.3 | | |

raphy showed that the reaction was complete and that only one product was present.

*2-Acylcyclohexane-1,3-diones I.* The enol ester (0.018 mol) was refluxed in dry toluene with 4-pyrrolidinopyridine (1 g, Aldrich) for 4 hr. After removal of the toluene by evaporation, the reaction mixture was taken up in chloroform and passed through a short Florisil column in chloroform to remove the catalyst. TLC analysis of the reaction mixture (chloroform–0.1% TFA) showed one major product and a number of less polar components. The product was purified by HPLC on a silica column with methylene chloride–0.1% TFA as eluent using the $\beta$-triketone absorption at 274 nm for detection. The yield of 2-acylcyclohexane-1,3-diones from the enol ester was typically 30%.

The chromatographic behavior (TLC and HPLC) and the [$^{13}$C]NMR (Table 1), [$^{1}$H]NMR, and EIMS data for all three 2-acylcyclohexane-1,3-diones were identical with those of the natural products (Mudd, 1981, 1983).

## DISCUSSION

The scheme involves synthesis, where necessary, of the appropriate acid corresponding to the acyl side chain and its conversion to the acid chloride. This disadvantages of direct *C*-acylation can be overcome by *O*-acylation of cyclohexane-1,3-dione forming the enol ester II which can then be rearranged to the 2-acylcyclohexane-1,3-dione I. Rearrangement of the enol ester with aluminium chloride (Akhrem 1978) works well for short saturated aliphatic ($C_1$–$C_6$) and aromatic side chains but when applied to the synthesis of 2-oleoylcyclohexane-1,3-dione, little or none of the desired product was obtained. However, rearrangement of the enol ester to the corresponding 2-acylcyclohexane-1,3-diones with 4-dialkylamino pyridines (Tanabe et al., 1982) proceeds smoothly in reasonable yield and preserves the geometry of the side-chain double bonds. 4-Pyrrolidinopyridine appears to be a slightly more effective catalyst than 4-dimethylaminopyridine; nevertheless, to avoid lengthy reaction times and possible side-chain degradation, a relatively large ratio of catalyst to enol ester was required.

All three synthetic 2-acylcyclohexane-1,3-diones caused similar oviposition responses from *Nemeritis* at the same dose (10 $\mu$g) as the corresponding natural kairomonal components (Mudd and Corbet, 1982).

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# VOLATILE FATTY ACIDS OF FRASS OF CERTAIN OMNIVOROUS INSECTS

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**Abstract**—The frass of the following omnivorous insects reared on natural and artificial diets was analyzed for volatile fatty acids: *Blattella germanica, Acheta domesticus, Blaberus discoidalis.* Acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were identified in all frass samples. The possible significance of volatile fatty acids in frass is discussed.

**Key Words**—Volatile fatty acids, frass, Dictyoptera, Orthoptera, *Blattella germanica, Acheta domesticus, Blaberus discoidalis.*

## INTRODUCTION

The volatile fatty acids—namely acetic, propionic, isobutyric, butyric, isovaleric and valeric acids—are produced naturally by microbial fermentation. They are the chief end-products of cellulose digestion in the rumen, in the hindgut of termites, and in larvae of the scarab beetle, *Oryctes nasicornis* (Breznak, 1982).

Recently, McFarlane et al. (1983) found these volatile fatty acids in the frass of the omnivorous *Acheta domesticus* (L.) fed on an artificial diet and showed that one of them—propionic acid—led to aggregation of larvae when placed on filter paper in the rearing jar. The amounts found were considerably less than found in the hindgut fluid of termites (Odelson and Breznak, 1983). The gut of *A. domesticus* has been shown to contain anerobic bacteria which ferment sugar to volatile fatty acids, but no cellulolytic bacteria were found (Ulrich et al., 1981). On the other hand, McFarlane and Distler (1982) have shown that *A. domesticcus* fed on an artificial diet will digest about 40% of ingested cellulose. The mechanism of cellulose dissimilation in *A. domesticus* is therefore unclear.

These results with *A. domesticus* have prompted an investigation of the frass



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of a number of omnivorous and phytophagous insects to see whether the production of volatile fatty acids is a normal feature of gut function. Possible effects on behavior of the volatile fatty acids are being investigated.

## METHODS AND MATERIALS

Frass was collected over a 24-hr period from larvae and adults of *Acheta domesticus* reared on an artificial diet (McFarlane and Distler, 1982), and from larvae and adults of *A. domesticus* reared on Ralston Purina rabbit chow. Frass was also collected over a 24-hr period from larvae and adults of both *Blattella germanica* (L.) and *Blaberus discoidalis* (Audinet-Serville) reared on Ralston Purina dog chow.

The major components of the artificial diet were casein (42.1%), "Alphacel" nonnutritive cellulose (31.6%), and D-(+)-dextrose (21.1%), all obtained from ICN Pharmaceuticals, Cleveland, Ohio.

*Analysis of Frass and Food for Volatile Fatty Acids.* Samples (80–300 mg) of frass or of food were extracted with distilled water; the extracts were filtered and the volume of the filtrate was adjusted to 25 ml. Preparation of the extracts for analysis of volatile fatty acids was performed according to the method described by Senshu et al. (1980). The extracts were neutralized by titrating against a 0.02 N NaOH solution (all extracts of frass were initially acid); a slight excess (1 ml) of NaOH solution was then added. The titrated extracts were then oven-dried (80°C). The sodium salts of the volatile fatty acids were dissolved in distilled water (2 ml), acidified with excess $H_2SO_4$ (30%, w/v), and analyzed immediately by gas–liquid chromatography.

Chromatography was carried out using a Varian gas chromatograph (model 3700) equipped with a flame ionization detector. The conditions of chromatography were as follows: glass column (1.8 m long, 2 mm internal diameter); column packing material—Chromosorb 101 (Chromatographic Specialities Limited, Brockville, Ontario, Canada); nitrogen carrier gas—flow rate = 20 cc/min; temperature programing—initial temperature = 150°C, initial hold = 3 min, program rate = 3°C/min, final temperature = 180°C, final hold = 12 min; injector temperature = 230°, detector temperature = 250°C. Individual peaks were identified by reference to a standard mixture of acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids.

## RESULTS

Analysis of various foods for volatile fatty acids is given in Table 1. The glucose component of the artificial diet contained the greater proportion of volatile fatty acids.

TABLE 1. VOLATILE FATTY ACIDS (g/100 g DRY WEIGHT) IN VARIOUS FOODS (% IN PARENTHESES)

| Acid | Food: Diet for *A. domesticus* | Rabbit chow | Dog chow |
|---|---|---|---|
| Acetic | 0.152 (77) | 0.092 (72) | 0.071 (68) |
| Propionic | 0.039 (20) | 0.010 (8) | 0.012 (11) |
| Isobutyric | 0.004 (2) | Tr | Tr |
| Butyric | Tr | 0.006 (5) | Tr |
| Isovaleric | 0.002 (1) | 0.018 (14) | 0.018 (17) |
| Valeric | ND | 0.001 (1) | 0.004 (4) |
| Total | 0.197 | 0.127 | 0.105 |

Analysis of the frass of *A. domesticus* fed artificial and rabbit chow diets is presented in Table 2. Analysis of the frass of *Blattella germanica* and *Blaberus discoidalis* fed dog chow is given in Table 3.

## DISCUSSION

All of the frass samples examined contained volatile fatty acids. Acetic acid was the most abundant fatty acid, followed generally by isovaleric acid and then propionic acid. The remaining three fatty acids were present in small or trace amounts. It is characteristic of microbial fermentation by mixed microbial communities that acetic acid is the most common volatile fatty acid produced. The

TABLE 2. VOLATILE FATTY ACIDS (g/100 g DRY WEIGHT) OF FRASS OF *A. domesticus* (% IN PARENTHESES)

| Acid | Reared on artificial diet | | Reared on rabbit chow | |
|---|---|---|---|---|
| | Larvae | Adults | Larvae | Adults |
| Acetic | 0.140 (74) | 0.149 (71) | 0.184 (68) | 0.193 (67) |
| Propionic | 0.022 (12) | 0.027 (13) | 0.024 (9) | 0.032 (11) |
| Isobutyric | 0.001 | 0.001 | Tr | Tr |
| Butyric | 0.004 (2) | 0.004 (2) | 0.010 (4) | 0.011 (4) |
| Isovaleric | 0.023 (12) | 0.028 (13) | 0.045 (17) | 0.042 (15) |
| Valeric | Tr | Tr | 0.008 (3) | 0.010 (3) |
| Total | 0.190 | 0.209 | 0.271 | 0.288 |

TABLE 3. VOLATILE FATTY ACIDS (g/100 g DRY WEIGHT) OF FRASS OF TWO COCKROACH SPECIES REARED ON DOG CHOW (% IN PARENTHESES)

| Acid | *Blattella germanica* larvae and adults | *Blaberus discoidalis* Larvae | *Blaberus discoidalis* Adults |
|---|---|---|---|
| Acetic | 0.097 (70) | 0.133 (60) | 0.108 (85) |
| Propionic | 0.016 (11) | 0.015 (8) | 0.010 (8) |
| Isobutyric | 0.0005 | Tr | Tr |
| Butyric | 0.002 (1) | 0.011 (6) | 0.001 (1) |
| Isovaleric | 0.019 (14) | 0.034 (18) | 0.008 (6) |
| Valeric | 0.005 (4) | 0.0005 | Tr |
| Total | 0.1395 | 0.1935 | 0.127 |

proportion of acetic acid generally found here was, however, less than in the termite (Odelson and Breznak, 1983).

The diets fed the insects also, for the most part, contained the volatile fatty acids. In fact, the artificial diet surprisingly contained more volatile fatty acids than either of the commercial diets. With *A. domesticus* fed the artificial diet, isovaleric acid was ten times as concentrated in the frass as in the food. The results indicate that this fatty acid at least is produced in the insect gut. The gut of *A. domesticus* contains bacteria producing acetic, propionic, butyric, isovaleric, and valeric acids (Ulrich et al., 1981). With the other omnivorous insects, the use of artificial diets, together with analyses of food, will be required to indicate a role for microbial fermentation. It may also be desirable to study the anerobic bacteria of the gut of these insects.

*A. domesticus* on an artificial diet produces about one third the amount of frass as food eaten (McFarlane and Distler, 1982). As the concentration of acetic acid is about the same in frass as in food, this suggests that acetic acid is utilized by the house cricket. A similar conclusion can be made from the results for *A. domesticus* fed rabbit chow, and *Blattella germanica* and *Blaberus discoidalis* fed dog chow, assuming a similar ratio of food eaten to frass produced. Acetic acid is actively transported from gut to hemolymph by the rectal epithelium in certain insects (Phillips, 1981).

There was little difference in the fatty acid composition of the larval and adult frass of *A. domesticus* fed the artificial diet or the rabbit chow. With *Blaberus discoidalis* fed dog chow, however, there was considerable difference in the proportions of fatty acids, particularly butyric and isovaleric acids. This indicates a metamorphic change in gut physiology.

Volatile fatty acids may be found in the frass of all omnivorous insects reared in the laboratory, if not in nature. McFarlane et al. (1983) have demonstrated an attractancy of *A. domesticus* to propionic acid and McFarlane (1984) has

shown a repellent effect of several volatile fatty acids with *Blattella germanica.* These effects may play a role in population density control.

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# USE OF *Lemna minor* L. AS A BIOASSAY IN ALLELOPATHY[1]

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**Abstract**—Investigations in allelopathy often require the use of a bioassay for evaluating limited quantities of potentially active growth regulators. A bioassay procedure was developed using *L. minor* grown in 1.5-ml aliquots of nutrient medium with and without allelochemicals in wells of 24-well tissue culture cluster dishes with loose-fitting lids. Tests using six replications per treatment with several flavonoid compounds and derivatives of coumarin, benzoic acid, and cinnamic acid demonstrated that the bioassay was capable of measuring inhibition at levels of compound ranging from 50 to 1000 μmol. Strongly inhibitory treatments were visible after 1 or 2 days. After 7 days of growth, frond number, growth rate, and dry weight were used to evaluate effects. The bioassay system is relatively simple, very sensitive, reproducible, and can be used for testing small amounts and dilute concentrations of unknowns which have been separated by chromatography.

**Key Words**—Bioassay, *Lemna minor*, allelochemical, allelopathy, duckweed.

## INTRODUCTION

Biochemical interference (allelopathy) occurs in a variety of plant communities, and allelopathic relationships have implications for management practices in agriculture (Klein and Miller, 1980; Einhelling, 1981; Putnam, 1983). Progress in studies of allelopathy is often limited by the capability for determining the biological activity of suspected allelochemicals. Bioassays are necessary tools in

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studying allelopathy, and the choice of bioassay that is used often dictates the interpretation of data where allelopathic activity may be involved.

Several problems are evident in using bioassays; these include the recognition that (1) species vary in sensitivity to a specific allelochemical, (2) sensitivity differences occur for the several phases of plant growth that may be used in a bioassay, and (3) the mode of action of the numerous potential allelochemicals may not be the same. Further complications arise because generally there is a limited amount of sample available for testing. Certainly this is true for suspected allelochemicals isolated with high-resolution separations using gas or high-pressure liquid chromatography (GLC or HPLC). Thus, there is currently a need for bioassays in allelopathy research that are sensitive and reproducible over a broad range of growth effects, yet require limited quantities of a sample. An ideal bioassay also would be easy to maintain and would require minimal time and equipment.

Mitchell and Livingston (1968) listed numerous assay methods illustrating the range of procedures developed for monitoring plant-growth-regulating substances. Seed germination tests have been applied routinely in allelopathy, although bioassays testing other phases of growth have also been useful. Germination tests are simple and can be executed with fairly small solution volumes. However, they are often less sensitive than bioassays testing a greater spectrum of metabolic processes. For example, the concentration threshold for inhibition of grain sorghum germination by several phenolic acids is 25 times higher than that for inhibition of seedling growth (Einhellig et al., 1983). Rietveld (1983) reported that long-term seedling growth was more sensitive to juglone than either seed germination or radicle elongation. The enhanced sensitivity of a seedling bioassay is desirable, but may not be practical because of the larger quantity of allelochemical and the longer time required for the test.

We determined the suitability of the aquatic macrophyte *Lemna minor* L., lesser duckweed, as a bioassay for allelochemicals. This small floating plant consists of a single leaf-like frond and a single root. Daughter fronds are propagated vegetatively and plants generally appear as rosettes of three or four fronds. Duckweed has been used as a bioassay for several growth regulators and in toxicological studies (Offord, 1946; Nickell and Finlay, 1954; Nickell and Celmar, 1965; Walbridge, 1977; Huber et al., 1982). However, to our knowledge, it has not been used in allelopathy studies, and minimizing the quantity of sample required has not been emphasized. The intent of our research was to develop procedures and test the feasibility of using the growth of *L. minor* as an indicator of the biological activity of small fractions of allelochemicals.

## METHODS AND MATERIALS

*Plant Materials.* The aquatic flowering plant *Lemna minor* L. strain 5, from axenic cultures, was used. The growth habit and reproduction of *Lemna* has been described by Hillman (1961a).

*Culture Conditions.* We used the basic E culture medium as modified from Hillman (1961b) and described by Cleland and Briggs (1967). In early studies we included sucrose and tartaric acid in the medium, but deleted them in later work. Stock cultures of *L. minor* were grown in cotton-stoppered 125-ml Erlenmeyer flasks with 50 ml of medium. The flasks were stocked with one to three fronds transferred by loop under aseptic conditions in a laminar flow hood. The stock flasks were maintained in a growth chamber at 28°C under constant light (236 $\mu E/sec/m^2$), provided by fluorescent and incandescent bulbs. After one week, a culture flask contained a sufficient number of plants for a bioassay.

*Experimental Procedure.* The E medium was prepared with known concentrations of allelochemicals and autoclaved. After cooling, 1.5 ml of the medium was pipetted into wells of a 24-well tissue culture cluster dish[4] (8.9 × 13.3 cm). Each treatment was replicated six times. In the experiments conducted without organic compounds in the medium, allelochemicals were added to the autoclaved medium before dispensing it into the culture dish wells. One *L. minor* rosette containing three or four visible fronds was placed in each well. The culture dishes were then closed and placed in the growth chamber. Open pans of water were provided to maintain a high humidity. Cultures were inspected daily and fronds were counted during the first several days of a bioassay to determine the growth of *L. minor.* On day 7, the plants were removed from the individual wells, the number of fronds was determined, and the plants were oven dried (24 hr, 90°C) and weighed. In addition to frond number and dry weight, the growth rate was calculated according to Hillman (1961a):

$$\frac{\text{Log}_{10}[\text{final frond no. (Fd)}] - \log_{10}[\text{initial frond no. (Fo)}]}{\text{No. days (d)}}$$

The data were subjected to analysis of variance and Duncan's multiple-range test.

When the bioassay was used to evaluate fractions from HPLC separations, the unknowns were dissolved in ethanol, and 5 $\mu l$ of this solution was added to the medium in a culture dish well. Appropriate solvent controls were included and six replications were used for a test.

## RESULTS AND DISCUSSION

The bioassay procedures that were found most practical utilized E medium minus sucrose and tartaric acid, and culture dish lids were partially lifted at least once a day to enhance gas exchange. The total growth and calculated growth rate of *L. minor* using this procedure is illustrated in Figure 1. This bioassay system permits extensive replication of tests for a potential allelochemical, and quantitative measurement of several parameters. Differences in appearance of the cultures are evident one or two days after treatment for substances that are strongly

[4]Costar™. Available through Bellco Glass Inc., 340 Edrudo Road, Vineland, New Jersey 08360.

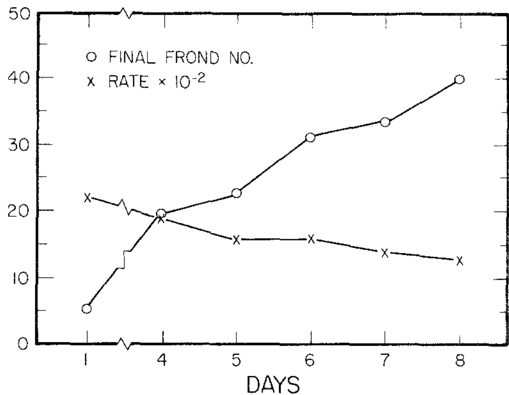


FIG. 1. Growth of *L. minor* during eight days in tissue culture cluster dish without organic amendment. Results are the average of six replications. The growth rate was calculated by the formula: $\text{Log}_{10}$ (final frond no.) -$\text{Log}_{10}$ (initial frond no.)/No. days.

inhibitory. Fronds may be chlorotic, have brown edges, be morphologically altered, or produce fewer daughter fronds. Treatments that are less inhibitory require more time to evaluate, but a growth period of 7 days provides good sensitivity.

These procedures detected inhibition of *L. minor* by concentrations of ferulic acid as low as 250 μM (Table 1). The frond margins were chlorotic 1 day after treatment with 1000 μM ferulic acid, and reduced frond production was apparent by the second day. Midway into the 7-day growth period, cultures treated with 500 μM ferulic acid had produced fewer fronds than controls. The data at harvest illustrate that even 250 μM ferulic acid significantly reduced the growth of *L.*

TABLE 1. EFFECTS OF FERULIC ACID (FA) ON GROWTH OF *L. minor* IN TISSUE CULTURE CLUSTER DISH WELLS[a]

| FA (μM) | Initial pH | Frond No. Initial | Frond No. Final | Growth Rate[b] (x $10^{-3}$) | Final dry wt. (mg) |
|---|---|---|---|---|---|
| — | 4.6 | 4.0 ± 0.3 | 55.6 ± 1.9a | 164 ± 3a | 4.9 ± 0.2a |
| 100 | 4.4 | 4.0 ± 0.0 | 50.5 ± 1.7ab | 157 ± 2ab | 5.3 ± 0.2a |
| 250 | 4.2 | 3.8 ± 0.2 | 43.2 ± 3.0b | 150 ± 4b | 4.1 ± 0.3b |
| 500 | 4.1 | 4.3 ± 0.2 | 26.5 ± 4.1c | 110 ± 6c | 2.9 ± 0.4c |
| 1000 | 3.9 | 5.1 ± 0.6 | 5.3 ± 0.7d | 1 ± 1d | 0.6 ± 0.1d |

[a]Values are the mean ± SE of six replications, 7 days of growth. Those in a column not followed by the same letter are significantly different, $P = 0.05$, ANOVA with Duncan's multiple-range test.
[b]Growth rate determined by the formula:

$$\frac{\text{Log}_{10}\text{(final frond no.)} - \text{Log}_{10}\text{(initial frond no.)}}{\text{No. days}}$$

*minor* based on the tissue dry weight. This level of sensitivity approximates that of the grain sorghum seedling bioassay and is superior to a sorghum germination bioassay (Rasmussen and Einhellig, 1977, 1979).

In developing the *L. minor* bioassay procedures, tests were conducted to determine the response of *L. minor* to variations in nutrient solution volume, pH, presence or absence of sucrose as an energy source, and amendments of ethanol. The effects of ethanol and pH were investigated because the introduction of a suspected allelochemical into the nutrient solution may alter pH, and in many cases it is necessary to test compounds that have been isolated in ethanol (Davis et al., 1978). Growth was reduced when the pH of the medium was below 4.0, but no changes were evident at pH values of 4.0–6.0, a range that permits testing many known allelochemicals. These findings do not preclude the fact that growth regulatory chemicals may vary in activity according to the pH of the medium (Blackman and Robertson-Cunninghame, 1953; Tanaka et al., 1982). Ethanol amendments amounting to 0.33 v/v in the nutrient medium did not alter the growth of *L. minor.* Thus, diluted ethanol may be used as a solvent for compounds that are to be tested with the bioassay.

The presence of tartaric acid and sucrose in the medium increases the potential problems in the bioassay because these organic compounds support growth of microorganisms in any cultures that become contaminated. They also may limit the sensitivity of *L. minor* to some allelochemicals, since the plants are not totally dependent upon photosynthesis as a source of carbon and energy. Although controversy exists over the importance of organic energy sources for growing *L. minor* (Hillman, 1961a), our results establish that sucrose and tartaric acid are not critical for maintaining stock cultures, or for the culture dish bioassay. This was demonstrated in tests that compared the effects of salicylic acid on *L. minor* with or without organic amendments (Figure 2). The control cultures without sucrose and tartaric acid grew as vigorously as did controls provided with these supplements, and prior maintenance of *L. minor* in stocks with the organics did not have an effect. The inhibition threshold for salicylic acid was 50 $\mu$M in the presence or absence of organic energy sources, but 100 $\mu$M treatments were more inhibitory when sucrose and tartaric acid were omitted from the *L. minor* stock cultures and the bioassay medium. Inhibition of *L. minor* growth by 50 $\mu$M salicylic acid indicates greater sensitivity than a sorghum seedling bioassay. Work in our laboratories has demonstrated that 100 $\mu$M salicylic acid is near the threshold for inhibition of sorghum seedlings, and the inhibition threshold is higher under some growth conditions (unpublished data).

The ferulic and salicylic acid data illustrate that for a particular test there is a close correlation between frond number and dry weight (Table 1 and Figure 2). The data from subsequent experiments reinforced this observation. In many cases bioactivity could be evaluated in the *L. minor* assay by determining frond number only. However this would not be sufficient if the allelochemical reduced frond size, but not number.

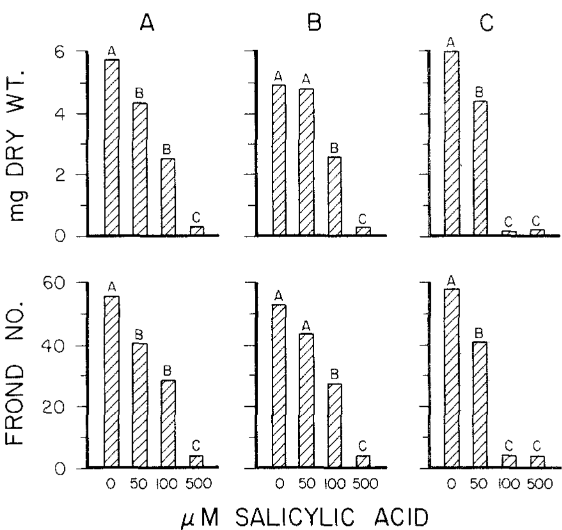


FIG. 2. Effects of salicylic acid on the average dry weight and final frond number per well of *L. minor* grown for seven days in tissue culture cluster dish. (A) Grown in E medium (see text for details). (B) Stock cultures in E medium and tests in E medium minus sucrose and tartaric acid. (C) Stock cultures and tests in E medium minus sucrose and tartaric acid. Bars within a graph having different letters are significantly different, $P = 0.05$, ANOVA with Duncan's multiple-range test.

Several flavonoids and derivatives of coumarin, benzoic acid, and cinnamic acid have been implicated as allelopathic chemicals in numerous situations (Rice, 1974, 1979; Putnam, 1983). We determined that the growth of *L. minor* was inhibited by 1000-$\mu$M concentrations of compounds selected from this group of allelochemicals. The frond number data from *p*-coumaric acid, *p*-anisic acid, esculetin, and catechin treatments illustrate these effects (Figure 3). Dry weight data reflected parallel effects (data not shown). We did not intend in these experiments to determine a precise threshold for inhibition, but the results demonstrate that inhibition by some compounds can be detected at concentrations well below 1000 $\mu$M. Other compounds tested were myricetin, rutin, scopoletin, umbelliferone, vanillic acid, *p*-hydroxybenzoic acid, and gallic acid. The growth of *L. minor* was inhibited by 50 $\mu$M salicylic acid. Other compounds were less inhibitory. The bioassay was also sensitive to stimulatory effects, as shown with the lowest concentrations of catechin (Figure 3). This feature adds a useful dimension to the utility of the bioassay.

Several procedural hints may be useful. The final frond count bears a definite relationship to the initial inoculation number, so it is important that reasonable consistency be maintained when fronds are placed in the wells to begin the bioassay. Each assay dish should have a control, and it is essential to randomize treatments. The lids of the culture dishes are loose fitting and, with periodic

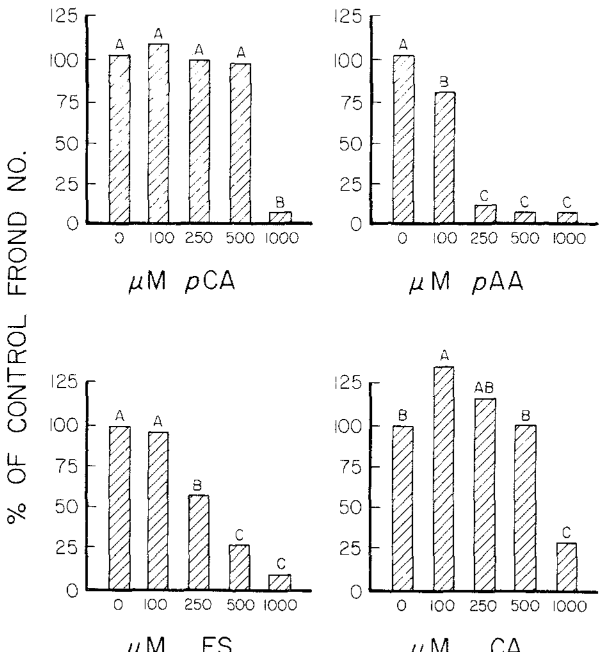


FIG. 3. Effects of *p*-coumaric acid (*p*CA), *p*-anisic acid (*p*AA), esculetin (ES), and catechin (CA) on the final frond number of *L. minor* grown for seven days in tissue culture dish wells. Bars within a graph having different letters are significantly different, $P$ = 0.05, ANOVA with Duncan's multiple-range test.

partial lifting of the lids, evaporative loss occurs from the culture dish wells. Those along the edges of the dish often lose the most solution, but this is not consistent. Loss of solution, plus frond crowding dictates limiting the growth period to 7 days. It could be shortened, but our tests with known allelochemicals demonstrated that a 4-day growth period is not as sensitive or selective as a 7-day period. Probably lower concentrations of inhibitory substances can be detected if more replications are utilized or if procedures are changed so that *L. minor* grows in a larger solution volume and for a longer time. However, such a change defeats the purpose cited for developing the bioassay. Although no effect of autoclaving on the allelochemicals was observed, we recommend sterilization of these chemicals by filtration when the E medium contains sucrose. Several allelochemicals reduced chlorophyll content of *L. minor*, and experiments are now in progress to determine the value of pigment analysis for increasing further the sensitivity of the bioassay.

We now routinely use *L. minor* for initial evaluations of the biological activity of fractions from HPLC separations of allelopathic extracts. By comparison to other evaluations we have attempted, the *L. minor* bioassay maximizes sensitiv-

ity, minimizes sample requirements, and provides a valuable preliminary aid to identifying natural products that may be the primary elicitors of allelopathy.

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# EFFECTS OF *Solanum* GLYCOALKALOIDS ON CHEMOSENSILLA IN THE COLORADO POTATO BEETLE
## A Mechanism of Feeding Deterrence?

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**Abstract**—Steroidal glycoalkaloids, found in species of the Solanaceae, elicit bursting activity in galeal and tarsal chemosensilla of adult Colorado potato beetles. The effect has an average latency of 6–12 sec, depending on the sensillum/alkaloid combination. A 20-sec alkaloid treatment is often sufficient to render galeal sensilla unresponsive to gamma-aminobutyric acid, normally an effective stimulant. The alkaloids have similar effects on galeal sensilla of larval Colorado potato beetles and on labellar chemosensilla of the blowfly. It is concluded that these compounds act independently of any specialized chemoreceptor in the Colorado potato beetle, and that association of the Colorado potato beetle with solanaceous plants has not led to evolution of a specific receptor for *Solanum* glycoalkaloids.

**Key Words**—Colorado potato beetle, *Leptinotarsa decemlineata,* Coleoptera, Chrysomelidae, feeding deterrents, Solanaceae, *Solanum* alkaloids, chemoreceptors, mouthpart sensilla, tomatine, solanine, chaconine, demissine, leptine III.

## INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is an oligophagous phytophagous insect which naturally feeds on several members of the family Solanaceae (Tower, 1918) and which can be reared experimentally on some nonsolanaceaous plants (Hsiao and Fraenkel, 1968). Among species of *Leptinotarsa* studied to date, it appears that *L. decemlineata* is one of the least specific in its host plant selection, and it has been suggested that this relative lack of specificity may have contributed to its status as a widespread pest (Hsiao, 1974).

The solanaceous host plants of *L. decemlineata* contain a number of related steroidal glycoalkaloids, with the genera *Solanum* and *Lycopersicon* being par-



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ticularly rich sources of these compounds (Schreiber, 1979). Since the mid-40s *Solanum* glycoalkaloids have been implicated as feeding deterrents for *L. decemlineata* (see Bongers, 1970, for an extensive list of references to early papers). Although many of the steroidal alkaloids from *Solanum* and *Lycopersicon* are chemically quite similar (Schreiber, 1979), there is evidence that they differ in their effect on the Colorado potato beetle. Stürckow and Löw (1961) compared potencies of a number of *Solanum* glycoalkaloids by infusing them into leaves of *Solanum tuberosum* and determining reduction in feeding on treated leaves by adult beetles. The leptins, which are acetylated glycoalkaloids (Sinden et al., 1980), were clearly the most potent. Among the nonacetylated glycoalkaloids, tomatine, a compound characteristic of species of *Lycopersicon* including tomato, and also present in some species of *Solanum*, was slightly more potent as a feeding deterrent than solanine or chaconine. The latter two compounds both occur commonly in species of *Solanum*, including *Solanum tuberosum* (Schreiber, 1979; Gregory et al., 1981).

Since these early studies, the relationship between species of *Solanum* and *L. decemlineata* has been explained largely on the basis of assumed differences in sensitivity of the beetles (larvae and adults) to the varied alkaloids in potential host plants. In this context the *Solanum* alkaloids have been variously referred to as phagorepellents (Levinson, 1976), repellents (Robinson, 1974; Levin, 1976), and deterrents (Hsiao, 1974, Sinden et al., 1978) for the Colorado potato beetle. Hsiao (1974) extended studies to include several species of *Leptinotarsa,* demonstrating that these species differed in their sensitivities to various commercially available solanaceous alkaloids, including steroidal glycoalkaloids.

In many of the above papers it is implied, although not often explicitly stated, that the alkaloids exert their effect directly via the chemosensory system of the beetle. Bongers (1970) was most explicit, stating that "while some of these alkaloids may be toxic, the effect of several others (e.g., tomatine) is a sensory inhibition of feeding activity."

Only one previous study has attempted to address this possibility directly. Stürckow (1959) investigated tarsal sensilla in adult beetles and described bursting activity from the cells when tomatine and solanine were applied. The delayed bursting activity in Stürckow's electrophysiological recordings could be interpreted as due to injury of the sensory cells (Städler, 1984; see Discussion). Also, it is difficult to see how these tarsal sensilla would normally come in contact with alkaloids, or that they would be the only means of detecting alkaloids.

In this study, we reinvestigate the hypotheses that chemosensory cells are responsible for detecting *Solanum* alkaloids that are potential feeding deterrents and for providing differential sensitivity to these chemically similar compounds. Sensilla on the maxillary galeae of adult beetles are accessible to electrophysiological recording (Mitchell and Harrison, 1984) and presumably are well situated for detecting chemical compounds inside the leaf as it is macerated by the mandibles. They also contain cells sensitive to amino acids and sucrose (Mitchell

and Harrison, 1984), making it possible to determine if the alkaloids have an inhibitory effect on responses to these feeding stimulants. We chose these sensilla as the focal point of this study. We also briefly tested larval galeal sensilla and adult tarsal sensilla of *L. decemlineata* and labellar sensilla of *Phormia regina* Meigen.

## METHODS AND MATERIALS

Larval and adult *L. decemlineata* were obtained from a colony maintained in our laboratory under conditions described in a previous paper (Mitchell and Harrison, 1984). Most of this colony is replaced annually from natural populations in the Edmonton area. Blowflies were obtained from a colony reared in the Department of Entomology.

The galeae of adult beetles are usually held close to neighboring mouthparts and access to them is obstructed by the mandibles. To record from galeal sensilla, a procedure for restraining the whole animal and exposing the galea by tying a hair around the palpus was used (Mitchell and Harrison, 1984). Tarsal sensilla on adult beetles are easily accessible with the restrained, whole animal preparation mentioned above. We therefore did not excise the leg but restrained it in a suitable position for recording. Recordings from larval galeae were made on excised head preparations after Mitchell and Schoonhoven (1974). The head was supported on a glass pipet filled with 0.1 M NaCl; this also served as the reference electrode. A similar excised head preparation was used for the blowfly recordings, which were made from large labellar sensilla.

Tip recordings were used exclusively, with 0.05 M NaCl the control stimulus and solvent for all other compounds. Further details of the recording setup for adult beetles can be found in Sutcliffe and Mitchell (1982).

Since the alkaloids tested often caused apparent injury to sensory cells (see Results), care had to be taken in stimulus presentation and in interpreting results. To guard against deterioration of the preparation, periodic applications of GABA, sucrose, or NaCl were made. We continued to record from the sensilla only if this response was normal. At least 3 min was allowed between stimulus applications.

Sensilla were tested as described above, and the number responding at each concentration was determined. Response latencies were measured with a stopwatch while replaying experiments recorded on magnetic tape. Since the variability of these latencies was large, this type of time measurement was considered adequate. In determining latencies, low-level activity (1 or 2 impulses per second) in the early seconds of stimulation was ignored, since this level of activity was not always distinguishable from that of the control. Thus, latency reflects the time between stimulus application and the first obvious burst of impulses from a single cell or from several cells.

Chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, and were used without further purification. Solanine, chaconine, and tomatine are poorly soluble in water but all will go into solution (1 mM) at pH 2–3. We therefore lowered the pH of 0.05 M NaCl solution with HCl, dissolved the glycoalkaloid, then increased pH to 5–5.6 with NaOH. Above pH 5.6 the glycoalkaloids precipitated. Saline treated similarly but with no alkaloid and at pH 5 had no discernable adverse effect on the sensilla, nor was it any more stimulating than the control solution of 0.05 M NaCl at pH 6.5. All variance is expressed as standard deviation.

## RESULTS

*Adult Galea–Steroidal Alkaloids.* All three of the steroidal glycoalkaloids studied, i.e., solanine, chaconine, and tomatine, caused irregular firing by several (probably all) cells in the sensillum. The firing pattern was often burstlike with the first burst occurring after a delay of seconds. Figure 1 shows segments of a typical recording. The first burst occurred after approximately 12 sec of continuous stimulation, although there was some sporadic firing of small-amplitude impulses before this. The initial burst was from a single cell (uniform impulse height) with other cells becoming active later. A single cell usually formed the first part of the first burst in all sensilla; however, the impulse height was not always the same relative to those of later impulses, so we do not believe that the same cell always initiated the burst.

Results for the application of the three steroidal alkaloids to adult galeal sensilla are summarized in Tables 1 and 2. Twenty-three percent of tested galeal sensilla showed activity to 0.05 mM chaconine, while no activity was detected with solanine and tomatine at this concentration. The lowest effective concen-

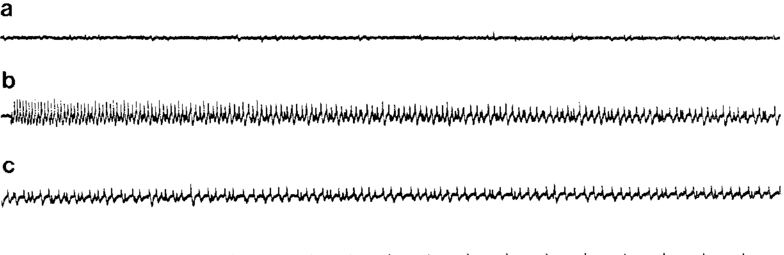


FIG. 1. Response to 0.2 mM solanine of a galeal sensillum of an adult Colorado potato beetle. (Sensillum is the alpha sensillum, i.e., the one that responds best to GABA, Mitchell and Harrison, 1984). Traces A, B, and C begin 12, 27, and 29.5 sec, respectively, after stimulus application. These are segments from a continuous record of a single stimulus application. Time marks on bottom at 0.1-sec intervals.

TABLE 1. PERCENT OF GALEAL AND TARSAL SENSILLA RESPONDING TO ALKALOIDS AT FIVE CONCENTRATIONS

| Conc. (mM) | Tomatine Galea | Tomatine Tarsi | Solanine Galea | Solanine Tarsi | Chaconine galea |
|---|---|---|---|---|---|
| 0.005 | | | | | 0 |
| 0.01 | 0 | | | | 7 |
| 0.05 | 0 | 0 | | 0 | 23 |
| 0.1 | 16 | 32 | 0 | 29 | 50 |
| 0.2 | 85 | | 30 | | 40 |
| 0.5 | 50 | 30 | 85 | 33 | 100 |
| 1.0 | 88 | 78 | 89 | 92 | 100 |
| Sensilla (*N*) | 43 | 30 | 34 | 21 | 35 |
| Animals (*N*) | 7 | 10 | 6 | 8 | 5 |

tration of tomatine was 0.1 mM, with 16% of tested sensilla showing activity. Solanine was ineffective until the concentration reached 0.2 mM, when 30% of tested sensilla showed activity. At 1 mM all three compounds induced activity similar to that shown in Figure 1 in all sensilla tested.

Latencies to all compounds were highly variable, ranging from less than 1 sec to 30 sec (Table 2). There was no significant difference between latencies for compounds on the galeal sensilla; these averaged between 9 and 12 sec. No clear dose–response relationship was found for any of the compounds.

Figure 2 shows recordings from an alpha galeal sensillum (Mitchell and Harrison, 1984) to GABA and 0.5 mM solanine. The response to GABA (trace A) was initially typical for an alpha sensillum but following treatment with 0.5 mM solanine for 20 sec (traces C and D), the response to GABA was completely abolished (trace B).

*Other Sensilla–Steroidal Alkaloids.* Results from our studies on the adult galea suggested an injury-related effect. If this is true, other chemosensitive cells would be expected to respond in a similar fashion. We chose adult Colorado potato beetle tarsal and blowfly labellar sensilla to test this hypothesis. Results

TABLE 2. RESPONSE LATENCIES (SEC ± SE) OF GALEAL AND TARSAL SENSILLA FOR ALL STIMULATING CONCENTRATIONS OF THREE ALKALOIDS[a]

| | Tomatine | Solanine | Chaconine |
|---|---|---|---|
| Galeal sensilla | 12 ± 9 | 9 ± 6 | 10 ± 7 |
| Tarsal sensilla | 15 ± 6 | 6 ± 3 | |

[a]Data are from pooled values. No differences in latencies were noted at different concentrations.

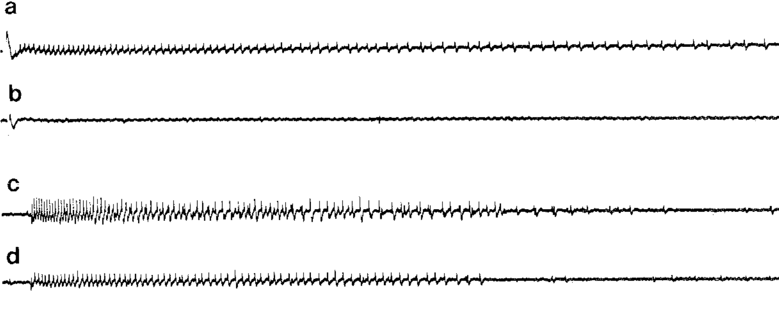


FIG. 2. Responses to 0.5 mM solanine and 10 mM GABA of a galeal sensillum on an adult Colorado potato beetle. (Sensillum is the alpha sensillum, i.e., the one that responds best to GABA, Mitchell and Harrison, 1984). Trace A, 10 mM GABA (initial response); trace B, 10 mM GABA following treatment with solanine; traces C and D, 0.5 mM solanine beginning 11 and 16 sec, respectively, after application. Time marks on bottom at 0.1-sec intervals.

for tarsal sensilla are summarized in Tables 1 and 2 for direct comparison with galeal sensilla. Only tomatine and solanine were tested. Both alkaloids caused activity in some sensilla at 0.1 mM, the lowest concentration tested, and at 1 mM most of the sensilla were active. Effects on tarsal sensilla were qualitatively similar to those on galeal sensilla, but tarsal sensilla appeared to be less sensitive on a population basis. There was no difference in sensitivity to solanine and tomatine. Solanine stimulated with a significantly shorter latency than tomatine ($P < 0.005$) (Table 2). Tarsal sensilla normally responded to 1.0 M NaCl at a rate of 20 $\pm$ 13 impulses/sec. After treatment with solanine and tomatine in various combinations for 1–5 sec, the average response to NaCl from 19 sensilla on six beetles fell to 5 $\pm$ 7.

Tomatine was tested on labellar sensilla of the blowfly to determine if it caused similar activity and injury in cells that do not naturally encounter these alkaloids. Results from one experiment are illustrated in Figure 3. The quality of the response to 1 mM tomatine was similar to that from galeal and tarsal sensilla of the Colorado potato beetle. Traces A, B, and C show segments of a typical response. The average latency on four sensilla was 14 $\pm$ 2 sec. In all cases, a low-impulse frequency (trace A) preceded the bursting response. Tomatine treatment also appeared to damage the sugar-sensitive cell in this preparation. Trace D shows a response to 0.5 M sucrose obtained at the beginning of this experiment and trace E the response to the same stimulus after tomatine treatment (traces A–C).

Cursory experiments were conducted with solanine and tomatine on Colorado potato beetle larval galeal sensilla. The effect was the same as described above for other sensilla.

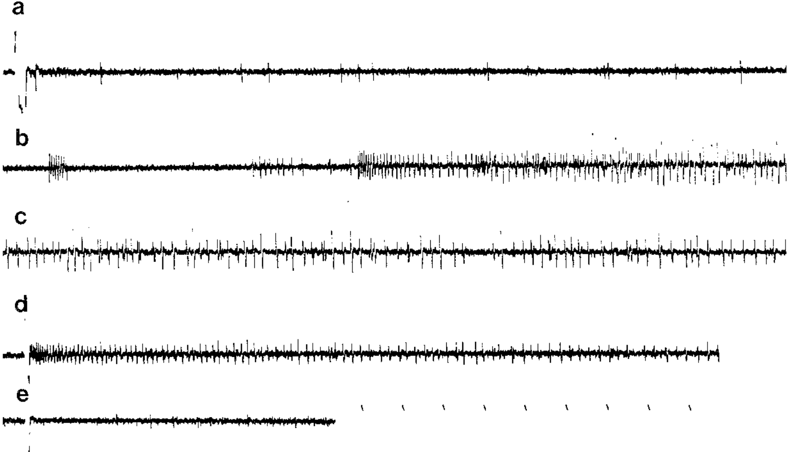


FIG. 3. Responses to 1 mM tomatine and 0.5 M sucrose of a labellar sensillum on an adult blowfly *P. regina.* Traces A, B, and C: 1 mM tomatine beginning at 0, 14, and 16 sec, respectively, from a continuous recording; trace D, 0.5 M sucrose (initial response); trace E, 0.5 M sucrose after tomatine treatment. Time marks at 0.1-sec intervals.

*Comparison with a Saponin.* Steroidal glycoalkaloids of solanaceous plants are biogenically related to and occur with saponins of similar structure. Both groups are $C_{27}$ steroids and are derived from cholesterol (Heftmann, 1967, 1973). Steroidal saponins reduce surface activity, a property used to partially explain their lytic effect on red blood cells (Segal et al., 1974). This surface activity is shared by steroidal alkaloids and was recognized by Schreiber (1958) as a possible mechanism by which these compounds could influence feeding in insects. We tested digitonin, a saponin related to tomatine, on sensilla of *P. regina* and the Colorado potato beetle. Its effects were similar to those of the glycoalkaloids. Figure 4 shows bursting activity during a continuous recording of stimulation with 1 mM digitonin on a galeal sensillum of an adult Colorado potato beetle

FIG. 4. Responses to 1 mM digitonin from labellar sensillum of *P. regina* (trace A) and galeal sensillum of the Colorado potato beetle (trace B). Traces A and B begin 3.4 and 14 sec, respectively, after stimulus application. The difference in overall activity of the two sensilla should not be interpreted as differences in sensitivity, but rather as an example of the variability of responses to these compounds.

(trace B) and a labellar sensillum of an adult blowfly (trace A). Digitonin treatment also severely reduced subsequent responses to sucrose in the blowfly. The threshold for digitonin bursting activity in galeal sensilla of the adult beetle was greater than 0.1 mM. More detailed studies have not yet been carried out on digitonin, but these results suggest that the actions of compounds like digitonin and tomatine are very similar on sensilla of plant feeding and non-plant-feeding insects.

## DISCUSSION

The results lead us to suggest that the mode of action of these compounds on these cells is of a general nature. That is, no specific receptors exist in the cells studied in Colorado potato beetles which provide sensitivity to these compounds. The strongest evidence in favor of this interpretation is the atypical bursting activity and injury (depolarization?) to the cells. Such effects should be general, and the similar effects on all three sensilla types tested, especially those on the blowfly, indicate that this is probably the case.

Stürckow (1959) found that 2% solanine caused bursting responses in tarsal hairs of the Colorado potato beetle, while 1% solanine did not. Comparing tomatine and solanine, she estimated the electrophysiological thresholds of the two compounds to be 0.1% (ca. 1.0 mM) and 1.5% (ca. 18 mM), respectively. The type of activity, judging from her published figures, was the same as we obtained on all sensilla studied here. She also found the response of tarsal sensilla to NaCl to be repressed by solanine. We observed no differences in the sensitivity of tarsal sensilla to tomatine and solanine, and a threshold of about 0.1 mM for both alkaloids. The 10-fold difference between solanine thresholds of tarsal sensilla in the two studies is difficult to explain. However, the fact that we also found little or no difference between tomatine and solanine in other sensilla suggests that there indeed may be no difference in the activity of the compounds at this level.

Behavioral studies comparing effects of steroidal glycoalkaloids on beetle feeding have been done by several authors. Stürckow and Löw (1961) studied five compounds and found that leptine III, an acetylated glycoalkaloid, was most potent. Table 3 summarizes some of their results. At the 50% feeding-inhibition level, there was no great difference among solanine, tomatine, chaconine, and demissine. Leptine III, however, was an order of magnitude more effective, leading the authors to conclude that the resistance of *Solanum chacoense* is due to its leptine content. The deterrent effect of tomatine on adults was also demonstrated by Sinden et al. (1978). They found that 1 mM tomatine infiltrated into tomato leaves could reduce feeding by 50–70%.

Larval Colorado potato beetles differ in their response to solanine and tomatine. Hsiao and Fraenkel (1968), and Hsiao (1974), using reduction in larval

TABLE 3. $ED_{50}$S (% WET WEIGHT) FOR FIVE STEROIDAL ALKALOIDS TESTED FOR DETERRENCE TO ADULT COLORADO POTATO BEETLES[a]

| Alkaloid | $ED_{50}$ (% net wt.) |
|---|---|
| chaconine | 0.6 |
| solanine | 0.4 |
| demissine | 0.4 |
| tomatine | 0.2 |
| leptine III | 0.02 |

[a]Values estimated from graphs presented by Stürckow and Löw (1961).

growth as a measure, showed that tomatine was approximately an order of magnitude more effective than solanine. However, such experiments do not clearly distinguish between effects on feeding behavior via the sensory system and toxic effects. Schreiber (1957) painted alkaloid solutions on potato leaves and found that tomatine and solanine had markedly different effects on larval feeding. He concluded that tomatine was effective, while solanine was ineffective as a feeding deterrent. He also investigated other steroidal alkaloids and suggested that those with an unsaturated steroid nucleus were not deterrent (e.g., solanine, chaconine) while saturated ones were deterrent (e.g., tomatine and demissin). Our results with solanine, chaconine, and tomatine failed to show such differences at the sensory level.

It seems that the steroidal glycoalkaloids as a group reduce feeding and slow development in adults and larvae, respectively. The implication has been that they act via the sensory system (see Introduction). Tomatine and solanine appear to differ in their effectiveness in behavioral bioassays. Is the sensory system involved in mediating the apparent deterrent effect? Does it distinguish between the steroidal glycoalkaloids?

If a sensillar field comes in contact with leaf juices containing steroidal alkaloids for sufficient time during feeding by adult beetles, then some of the sensilla would fire in bursts and possibly be damaged and no longer be sensitive to feeding stimulants. This combination of events could lead to a sensory-based inhibition of feeding. We have attempted to monitor galeal placement during feeding using video techniques. So far there is no conclusive evidence that the galeal sensilla are inundated with leaf juice for periods of time greater than 1 or 2 sec (unpublished observations).

Adult beetles also possess epipharyngeal sensilla. Three types are present, and one type has a visable pore in the tip. There are approximately 20 of these uniporous sensilla distributed over the central surface of the epipharynx (unpublished SEM observations). We have not been able to record from them, but observations presented above reveal no reason to expect that they would not be

sensitive to glycoalkaloids. Although these sensilla are probably almost continuously wetted by leaf juice during feeding, this would be difficult to demonstrate.

The different potencies of the nonacetylated steroidal glycoalkaloids in behavioral bioassays cannot be attributed to differential sensitivity at the sensory level on the basis of our results. The slightly higher potency of chaconine on galeal sensilla (Table 1) does not help resolve this, since chaconine was the least effective compound tested by Stürckow and Löw (1961) (Table 3). Results from a more recent study (Sinden et al., 1980) suggest that total glycoalkaloid content (TGA) may be a factor in determining amounts of damage caused by Colorado potato beetle feeding in field trials. This was particularly apparent in a group of eight clones of *Solanum chacoense* containing solanine/chaconine or dihydrosolanine/dihydrochaconine. The sensory results reported here support the idea that TGA levels are partly responsible for observed differences in host-plant acceptance. The galeal sensilla do not appear capable of differentiating among the three alkaloids tested here, and it remains to be seen if the epipharyngeal sensilla can do this.

## CONCLUSION

The steroidal glycoalkaloids clearly damage insect chemosensilla. Responses to them are burstlike and delayed in all sensilla tested. They probably act independently of any specialized receptor, and there is no evidence that the association of Colorado potato beetles with solanaceous plants has led to the evolution of a specific receptor for *Solanum* glycoalkaloids. If the chemosensory system is one of the mediators of feeding inhibition by these compounds, it is likely to be of the unspecific variety discussed by Schoonhoven (1982) as one of five possible mechanisms by which feeding deterrents can act. This includes compounds which affect nerve membranes by fortuitous association with polarization maintenace and/or receptor–effector mechanisms, leading to inactivity, bursting, or sporadic firing. Such unnatural "messages" could lead to rejection behavior by the insect. Mitchell and Sutcliffe (1983) suggest that these kinds of unspecific actions may be the major way in which plant secondary compounds influence insect chemosensilla, resulting in feeding deterrence.

With the evidence to date, the differential sensitivity to these compounds revealed by behavioral bioassays cannot be explained by a model that includes only chemosensilla. Some of these organs are likely involved in the insect's general sensitivity to total glycoalkaloid content of the plant, but other systems will likely have to be studied before all of the behavioral results can be satisfactorily explained.

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# DIFFERENCES AND SIMILARITIES IN CARDENOLIDE CONTENTS OF QUEEN AND MONARCH BUTTERFLIES IN FLORIDA AND THEIR ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

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**Abstract**—Florida queen butterflies are highly variable in cardenolide content and, in three populations studied, contained less cardenolide than did a sample of sympatric Florida monarchs. The possibility that queens stored a more potent set of cardenolides from their host plants (and therefore were as well protected as monarchs, even at lower concentrations) is refuted by chromatographic analysis of wild butterflies, as well as controlled laboratory rearings. It therefore appears that, with respect to cardenolides, monarchs are better defended than are queens. Consequently, cardenolides are unlikely to explain the apparent shift in Florida viceroy mimicry away from resemblance of the monarch, toward mimicry of the queen. Other hypotheses to explain this mimetic phenomenon are suggested. Adult monarchs exhibit significant negative correlations between the concentration of cardenolide stored in their tissues and both body size and weight, whereas queens show no such correlations. The implications of these results for the study of "metabolic costs" of allelochemic storage are discussed. Chromatographic evidence is provided that monarchs do breed in south Florida during the winter months and that the likely host plant employed by the population studied was *Asclepias curassavica.* This represents the first practical application of cardenolide "fingerprinting" to identify the larval host plants of wild danaid butterflies.

**Key Words**—*Danaus gilippus, Danaus plexippus,* Lepidoptera, Danaidae, cardiac glycosides, cardenolides, *Asclepias,* Asclepiadaceae, allelochemics, plant secondary chemistry, chemical ecology, chemical defense, mimicry, *Limenitis archippus.*

## INTRODUCTION

The butterfly family Danaidae has figured prominently in the development of the theory of plant-herbivore coevolution (Brower and Brower, 1964; Ehrlich

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and Raven, 1964) and has provided one of the best examples of the chemical defenses of insects against vertebrate predators (Brower, 1969; Brower et al., 1982; Dixon et al., 1978; Reichstein et al., 1968). However, much of our understanding of the ecology and evolution of plant–danaid–predator interactions is based upon only one species in this tropical family, the monarch butterfly (*Danaus plexippus* L.; review in Brower, 1984). Because this species is known to be unique among danaids in certain other respects (e.g., migration: Urquhart, 1960; lack of plant-derived sex pheromones: Edgar et al., 1976; Boppré, 1978), one must question whether monarchs are representative of the Danaidae with respect to host plant adaptation and chemical defense, or whether they represent just one point (or range) within a broader spectrum of danaid adaptations. If such a spectrum is found to exist, it may ultimately become possible to reconstruct some of the evolutionary steps leading to the close association between danaids and their highly toxic milkweed host plants (Asclepiadaceae).

Danaid species differ in the amounts of toxic cardenolides which they sequester from their food plants. For example, Brower et al. (1975) showed that the cardenolide concentrations of laboratory-reared African queen butterflies (*Danaus chrysippus* L.) were only about 30% that of monarchs simultaneously reared on the same milkweed host plants (see also Brower et al., 1978; Rothschild et al., 1975). The cardenolide concentrations of queen butterflies (*D. gilippus berenice* Cramer) from Florida were, on average, about 75% that of monarchs reared in the laboratory on the same host plants. Other genera of danaids (e.g., *Amauris, Euploea*) appear to prefer as larval host plants those milkweed species lacking cardenolides (Rothschild and Marsh, 1978).

Does this diversity in the storage of cardenolides reflect differences among danaid species in their degree of adaptation to these defensive plant allelochemics? Do queens, for example, store less cardenolide than monarchs because they are less well adapted and can tolerate these compounds less readily? Do the cardenolide contents of various danaid species differ only quantitatively, as described above, or are there also qualitative differences in the particular set of host plant cardenolides sequestered?

Here these questions are addressed through a comparison of the cardenolide contents of wild queen butterflies (*D. gilippus berenice*) from three populations in Florida and of monarchs (*Danaus plexippus*) from one of these populations. Following Brower and Moffitt (1974), the "cost" of storing cardenolides will be assessed by searching for correlations between body size or weight and cardenolide concentration of the butterflies. Since body size and weight are typically correlated with fecundity in Lepidoptera (see Hinton, 1981), a negative correlation with cardenolide concentration would suggest that one component of fitness (i.e., fecundity) has been traded for another (e.g., higher survival due to chemical defense from cardenolides). While such a trade may well be of positive net value to the insect, it nevertheless would require an investment or cost which, it is assumed, should be lessened as adaptation to allelochemics evolves.

The subspecies of queen studied here is of further interest because it is the apparent model for mimicry by the southern subspecies of the viceroy butterfly (*Limenitis archippus floridensis* Strecker), which throughout the remainder of North America mimics the monarch (Brower, 1958a,b; Klots, 1951; Remington, 1968). Thus, a comparison of the chemical basis for defense in the queen and monarch should aid in understanding both the selective rationale for the switch in viceroy mimicry and the broader issues of herbivore adaptation to host plants mentioned above.

## METHODS AND MATERIALS

Wild queen butterflies were collected from three populations in Florida, listed from north to south as follows: Lake Istokpoga, Highlands County, September 7, 1981; Corkscrew Swamp Sanctuary, Collier County, September 6, 1981; and Miami, Dade County, December 4, 1981. In addition, monarchs were collected from the Miami site where they were sympatric with queens, and where only the milkweed *Asclepias curassavica* grew abundantly. (However, a few *A. incarnata* plants were also located.) These collections therefore permit a study of population variation in cardenolide content of queens, as well as a comparison of the relative value, with respect to cardenolides, of monarchs and queens as models for viceroy mimicry. All butterflies were placed on ice immediately following capture, killed by freezing, and later dried for 16 hr at 60°C. Dry weights were determined using a Mettler AK-160 electronic balance. The right wing was removed with forceps, and the distance from the apex to the anterior notal process was measured to the nearest 0.5 mm with hand calipers. Fat was removed from the butterflies by petroleum-ether extraction of each entire insect for 1 hr (methods in Walford and Brower, 1985). This procedure removes only negligible amounts of cardenolide from the insect (Nelson and Brower, unpublished data; see also Nishio, 1980). Lean weights were calculated by subtracting the weight of extracted fat from the total dry weight of each insect.

Cardenolide content was determined by standard spectrophotometric methods (Brower et al., 1972, 1975), with one modification. Soon after beginning the spectroassay of queens from Corkscrew Swamp, it became evident that many of the butterflies contained very little cardenolide. In such cases it is frequently difficult to achieve a stable absorbance reading. Consequently, 0.3 ml of a $12.5 \times 10^{-5}$ M ethanolic solution of digitoxin was added to each butterfly extract in the cuvette (replacing 0.3 ml of 95% ethanol; see Brower et al., 1972) in order to artificially "boost" absorbance readings to a more stable mid-range. A pilot test demonstrated that the absorbance of the "boost" digitoxin alone was $0.400 \pm 0.009$ ($\bar{X} \pm$ SD; $N = 9$). This mean value was therefore subtracted from the total absorbance read for a sample, the remainder being the absorbance due to the butterfly extract alone. In order for this remainder to be considered signifi-

cantly different from zero, it had to exceed background level (0.400) by at least two standard deviations (i.e., 0.018); thus, the minimum detectable cardenolide concentration, using this method was 3 μg/0.1 g.

In order to compare qualitatively the cardenolides present in sympatric monarchs and queens from the Miami sample, those butterflies containing a total of at least 30 μg equivalents of digitoxin (as determined from the spectroassay) were subjected to a lead acetate clean-up procedure in preparation for thin-layer chromatography (TLC). The procedure used was that described by Brower et al. (1982) with the exception that the final solution was filtered through a Millipore filter (Millipore Corp., Bedford, Massachusetts) rather than through a funnel of glass wool and anhydrous sodium sulfate. This clean-up procedure removes much of the interfering pigments and other noncardenolide compounds from the butterfly samples. TLC was then performed and plates developed four times in a chloroform–methanol–formamide solvent system (90:6:1). Further details of the TLC procedure are available in Brower et al. (1982).

In addition to the wild-caught butterflies, eggs and first instar larvae of both monarchs and queens were collected from milkweed plants growing in La Vega province, Dominican Republic, during July 1981. These were brought back to the laboratory and reared to maturity on an exclusive diet of *A. humistrata* leaves, collected wild in the vicinity of Gainesville, Florida. The leaves of this species contain relatively high concentrations of cardenolide (Cohen and Brower, 1982). From these rearings, 10 adult monarchs and seven queens were compared for quantitative (via spectrophotometry) and qualitative (via TLC) differences in cardenolide storage. For comparison, two arctiid moth species (*Cycnia tenera,* an apocynale specialist; and *Estigmene acraea,* a highly polyphagous species; Tietz, 1972) were reared on *A. humistrata* and chromatographed along with the danaids.

## RESULTS

*Wild-Caught Butterflies from Florida.* The frequency distributions of cardenolide concentration and total cardenolide per insect for the the wild-caught butterflies are shown in Figures 1 and 2, respectively. Each distribution departs significantly from normality [Shapiro-Wilk ($W$) tests; $P < 0.01$; Helwig and Council, 1979]. Thus, nonparametric statistical analyses were used.

Males and females did not differ significantly either in cardenolide concentration or total content in any of the queen or monarch populations studied (Table 1; Wilcoxon two-sample tests, $P > 0.05$ for all pairwise comparisons). Consequently, the data from both sexes of each population were pooled for all subsequent analyses.

Cardenolide concentration varied significantly among the three queen populations (Kruskal-Wallis $H = 31.60$, $df = 2$, $P < 0.0001$), as did the total

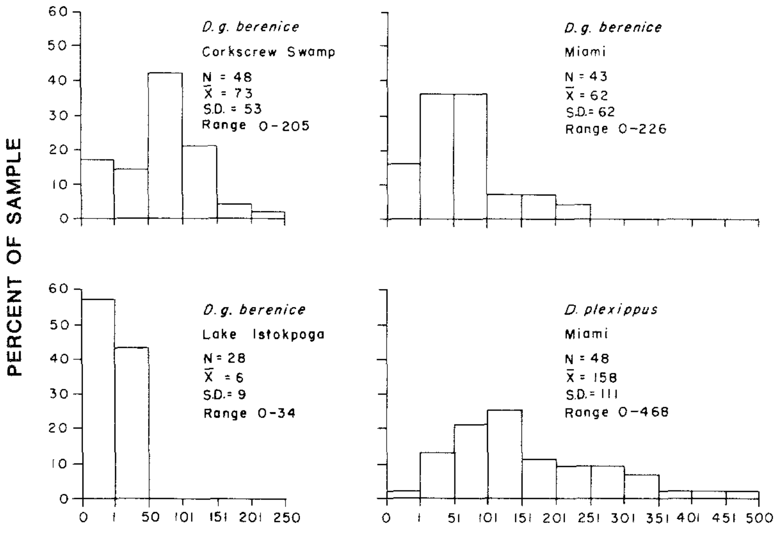


FIG. 1. Frequency distributions of cardenolide concentration (sexes pooled) for three populations of Florida queen butterfly, and one population of monarchs. Units in digitoxin equivalents.

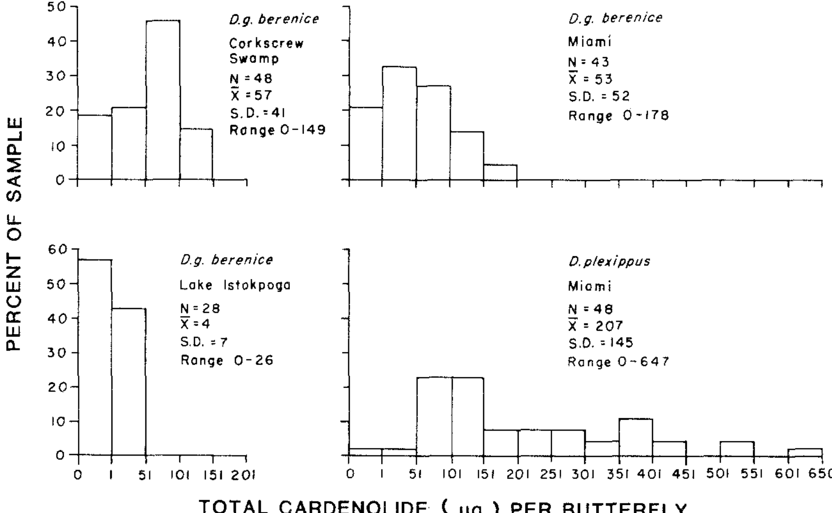


FIG. 2. Frequency distributions of total cardenolide per individual butterfly (sexes pooled).

TABLE 1. RIGHT WING LENGTHS, WEIGHTS, FAT, AND CARDENOLIDE CONTENTS OF WILD-CAUGHT QUEEN AND MONARCH BUTTERFLIES FROM THREE SITES IN FLORIDA: LAKE ISTOKPOGA (LI), CORKSCREW SWAMP (CS), AND MIAMI (MI)

| Species (site) | Sex | $N$ | Wing length (cm) | | Dry weight (mg) | | Lean weight (mg) | | Percent fat | | Cardenolide: Concentration ($\mu$g/0.1 g) | | Cardenolide: Total per insect ($\mu$g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD |
| Queens | | | | | | | | | | | | | | |
| LI | M | 15 | 4.22 | 0.19 | 94.9 | 22.8 | 84.4 | 19.4 | 10.9 | 3.0 | 8 | 11 | 6 | 9 |
| | F | 14 | 3.99 | 0.27 | 87.8 | 22.6 | 78.9 | 20.4 | 10.1 | 3.6 | 4 | 5 | 3 | 4 |
| CS | M | 21 | 4.27 | 0.22 | 95.2 | 16.4 | 88.8 | 15.3 | 6.7 | 2.4 | 64 | 44 | 57 | 42 |
| | F | 27 | 4.08 | 0.25 | 84.5 | 15.8 | 77.1 | 14.0 | 8.6 | 1.8 | 79 | 59 | 58 | 42 |
| MI | M | 26 | 4.26 | 0.18 | 92.4 | 15.1 | 82.9 | 12.1 | 10.0 | 3.2 | 78 | 71 | 66 | 58 |
| | F | 19 | 4.24 | 0.21 | 107.4 | 31.6 | 87.2 | 16.0 | 16.4 | 10.2 | 37 | 35 | 34 | 31 |
| Monarchs | | | | | | | | | | | | | | |
| MI | M | 25 | 4.92 | 0.29 | 146.6 | 28.1 | 136.6 | 23.9 | 6.4 | 2.6 | 150 | 90 | 198 | 116 |
| | F | 23 | 4.89 | 0.29 | 158.8 | 42.4 | 133.5 | 25.4 | 14.0 | 9.6 | 166 | 131 | 216 | 173 |

cardenolide content per butterfly ($H = 30.03$, $df = 2$, $P < 0.0001$). Pairwise comparisons revealed that the queens from Lake Istokpoga had significantly lower cardenolide concentrations than either those from Corkscrew Swamp (Wilcoxon two-sample test; $Z = 5.17$, $P < 0.0001$) or those from Miami ($Z = 4.72$, $P < 0.0001$), but that the latter two populations did not differ significantly from one another ($P > 0.05$). Similar results were found for total cardenolide content per butterfly.

Analysis of the Miami samples shows that monarchs had significantly greater cardenolide concentrations (Wilcoxon two-sample test; $Z = 4.82$, $P < 0.0001$) and total contents ($Z = 6.12$, $P < 0.0001$) than the sympatric queens. Chromatography reveals a single cardenolide profile common to both species (Figure 3). This consists of nine spots, including a major one at the approximate $R_f$ of digitoxin, and one of higher $R_f$. A few individuals (of both species) exhibit all of these spots plus an additional two faint spots of still higher $R_f$ (numbered spots 10 and 11 in Figure 3). This chromatogram is virtually identical to that of monarchs reared in the laboratory on *Asclepias curassavica* (Figure 3 inset), providing strong evidence that this was the host plant utilized by the Miami monarchs and queens. To date, no other milkweed species is known to produce this particular chromatographic profile in danaid butterflies (Brower et al., 1982, 1984a,b).

The wing lengths, dry and lean weights, and fat contents of the butterflies are summarized in Table 1. The correlations between these variables and cardenolide concentration are indicated in Table 2. For both male and female monarchs, there was a highly significant negative correlation between wing length and cardenolide concentration (Table 2C). Moreover, males also showed negative correlations between cardenolide concentration and both body weight and fat content. For queens, none of the correlations was significant in either sex (all three populations pooled; Table 2A).

These statistical differences between monarchs and queens could reflect true species differences. However, the monarchs were all collected in the Miami region, while the queens for this analysis were pooled from three areas. It is possible that an unknown geographic effect is operating here, such that butterflies from the Miami region, regardless of species, would show these negative correlations (e.g., due to a common host plant). To test this, the queens from Miami were also analyzed separately from the other two populations (Table 2B). However, as before, no significant correlations emerged ($P > 0.10$ for all tests). Thus, the observed species difference is not attributable to a geographic difference.

It is possible that monarchs show negative correlations between body size and cardenolide concentration, while queens do not, simply because they store greater concentrations of these chemicals (i.e., queens may not store sufficient cardenolide to be adversely affected by it). If this is the case, then the negative correlations should vanish for that subset of monarchs having concentrations

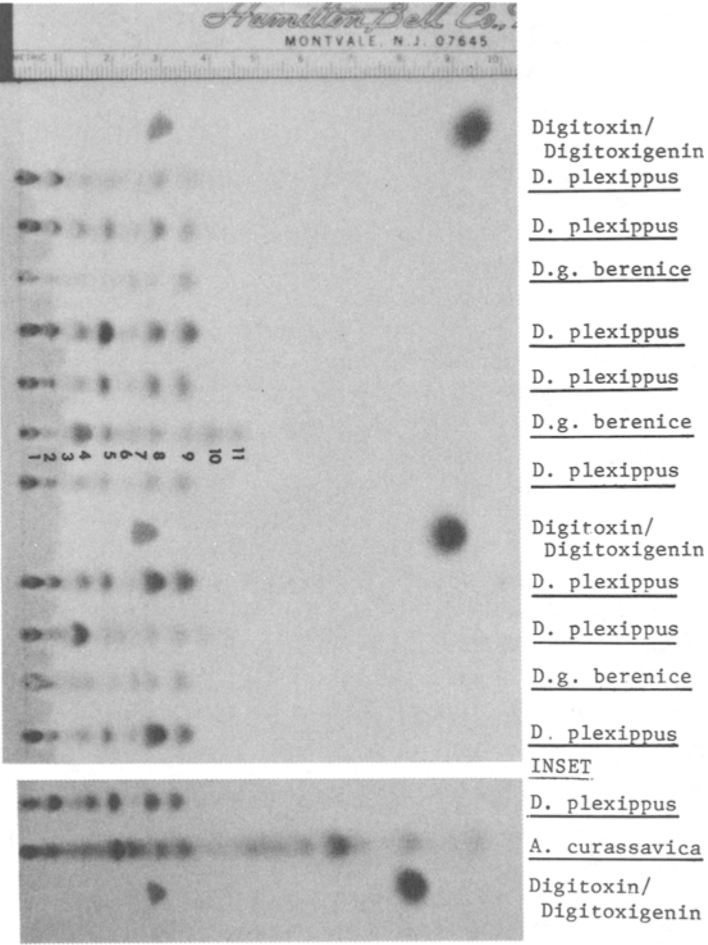


FIG. 3. Thin-layer chromatogram of wild-caught monarchs and queens from Miami area. Developed four times in chloroform–methanol–formamide (90:6:1 v/v/v). The great concordance of all profiles below spot 10 suggests that these animals had all fed upon the same larval host plant species. Note the agreement of this profile with that of monarchs reared in the laboratory on *Asclepias curassavica* (inset), a milkweed growing abundantly in the collection area. Since nearly all milkweed species studied to date produce qualitatively different TLC profiles in danaids (Brower et al., 1982, 1984a,b; see also Figure 4), *A. curassavica* is the likely larval host plant of the Miami butterflies. For reference, a 1:1 (v/v) mixture of digitoxin and digitoxigenin was spotted in three channels. Numbers to the right of each channel indicate the micrograms of cardenolide spotted, calculated prior to lead acetate clean-up. (inset from Brower, 1984)

TABLE 2. SPEARMAN CORRELATION COEFFICIENTS FOR CARDENOLIDE CONCENTRATION VS. BODY SIZE, WEIGHT, AND FAT CONTENT OF WILD-CAUGHT QUEEN AND MONARCH BUTTERFLIES FROM FLORIDA[a]

| | | | Cardenolide concentration versus | | | |
|---|---|---|---|---|---|---|
| Sample | Sex | $N$ | Wing length | Dry weight | Lean weight | Percent fat |
| Queens | | | | | | |
| A. All populations | Both | 119 | −0.004 | 0.01 | 0.03 | −0.14 |
| | M | 61 | −0.13 | 0.14 | 0.13 | −0.02 |
| | F | 58 | 0.03 | −0.08 | −0.08 | −0.25 |
| B. Miami only | Both | 43 | −0.17 | 0.20 | 0.20 | 0.14 |
| | M | 26 | −0.25 | 0.26 | 0.25 | 0.32 |
| | F | 17 | −0.20 | 0.32 | 0.23 | 0.14 |
| Monarchs | | | | | | |
| C. Entire Miami sample | Both | 48 | −0.53***[b] | −0.22 | −0.21 | −0.25 |
| | M | 25 | −0.55** | −0.44* | −0.39 | −0.53** |
| | F | 23 | −0.50* | −0.04 | −0.10 | −0.06 |
| D. Concentrations less than 226 μg/0.1 g | Both | 35 | −0.40* | −0.33* | −0.43** | −0.18 |
| | M | 18 | −0.36 | −0.46* | −0.49* | −0.36 |
| | F | 17 | −0.38 | −0.20 | −0.40 | −0.06 |

[a]The data for queens are first shown for all three populations pooled (A), and then for the Miami sample separately (B). Monarchs were first analyzed using the entire data set (C) and then by truncating the set such that only individuals having cardenolide concentrations equal to, or less than, that of the most concentrated queen (226 μg/0.1 g) were included (D). See text for explanation.
[b]$*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.0001$.

similar to those of queens. To test this, I analyzed only those monarchs (sexes pooled) having cardenolide concentrations equal to, or less than that of, the most highly concentrated queen (i.e., 226 μg/0.1 g). In this case, significant negative correlations between cardenolide concentration and wing length, dry weight, and lean weight still occurred (Table 2D). Thus, it appears that the difference between monarchs and queens is not due merely to geographic or cardenolide concentration differences. It is also not due to differences in larval host plant species, since Figure 3 demonstrates that both species in Miami had most likely developed on *A. curassavica.* Rather, the negative correlations appear to represent inherent species differences.

*Laboratory-Reared Butterflies (Dominican Republic Stock).* When monarchs were reared in the laboratory on *Asclepias humistrata,* the females developed

TABLE 3. BODY SIZES, WEIGHTS, FAT, AND CARDENOLIDE CONTENTS OF QUEENS AND MONARCHS (DOMINICAN REPUBLIC STOCK) REARED IN LABORATORY ON MILKWEED, *Asclepias humistrata*

| | | | | | | | | | | | Cardenolide | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Wing length (cm) | | Dry weight (mg) | | Lean weight (mg) | | Percent fat | | Concentration ($\mu$g/0.1 g) | | Total per insect ($\mu$g) | |
| Species | Sex | $N$ | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD | $\overline{X}$ | SD |
| Queen | | | | | | | | | | | | | | |
| | Both | 7 | 3.60 | 0.14 | 77.3 | 9.0 | 73.5 | 9.0 | 4.9 | 0.9 | 368 | 58 | 271 | 61 |
| | M | 3 | 3.63 | 0.12 | 82.2 | 7.7 | 78.6 | 8.0 | 4.5 | 1.1 | 341 | 34 | 269 | 55 |
| | F | 4 | 3.58 | 0.17 | 73.5 | 9.0 | 69.7 | 8.6 | 5.2 | 0.8 | 388 | 68 | 272 | 74 |
| Monarch | | | | | | | | | | | | | | |
| | Both | 10 | 4.55 | 0.18 | 147.8 | 31.6 | 140.3 | 30.8 | 5.1 | 3.1 | 489 | 145 | 657 | 156 |
| | M | 4 | 4.72 | 0.05 | 177.0 | 28.9 | 167.8 | 29.9 | 5.2 | 4.9 | 385 | 146 | 615 | 154 |
| | F | 6 | 4.43 | 0.12 | 128.4 | 12.8 | 122.1 | 12.8 | 5.0 | 1.7 | 558 | 104 | 684 | 164 |

TABLE 4. SPEARMAN CORRELATION COEFFICIENTS FOR CARDENOLIDE CONCENTRATION VS. BODY SIZE, WEIGHT, AND FAT CONTENT OF QUEEN AND MONARCH BUTTERFLIES (DOMINICAN REPUBLIC STOCK; BOTH SEXES POOLED) REARED IN LABORATORY ON *Asclepias humistrata*

| | | Cardenolide concentration versus | | | |
|---|---|---|---|---|---|
| Sample | $N$ | Wing length | Dry weight | Lean weight | Percent fat |
| Queens | 7 | 0.33 | 0.18 | 0.25 | −0.57 |
| Monarchs | 10 | −0.63**[a] | −0.56* | 0.05 | −0.58* |

[a] *$0.05 < P < 0.10$; **$P < 0.05$.

significantly higher cardenolide concentrations than did males (Wilcoxon two-sample test; $Z = 2.02$, $P < 0.05$; Table 3). No such sex difference was evident for queens reared under identical conditions ($Z = 0.53$; $P > 0.50$). As in the wild-caught samples, only the monarchs showed significant (again, negative) correlations between cardenolide concentration and other size and weight parameters (Table 4).

Thin-layer chromatography (Figure 4) demonstrates that the two danaid species (as well as two arctiid moth species) sequestered virtually identical sets of cardenolides from *A. humistrata.* This TLC profile is clearly distinguishable from that of butterflies reared on *A. curassavica* (cf. Figure 3 inset).

## DISCUSSION

*Body Size and Cardenolide Content.* Brower and Moffitt (1974) reported a negative correlation between the body weight and cardenolide concentration of female monarchs from Massachusetts and suggested that these individuals may have suffered a "metabolic cost," in terms of growth, of sequestering cardenolides for defense (see also Brower et al., 1972). Such negative correlations were not found for males from Massachusetts (which were 9% lower than females in mean cardenolide concentration), or in either sex collected in California (which were 62% lower in mean concentration than the Massachusetts females). However, these data confound sexual and geographic differences with correlated cardenolide concentration differences. Here, I have shown that negative correlations between cardenolide concentration and various size and weight parameters, which occur in monarchs but not in queens, are independent of geographic, sexual, food plant, or correlated concentration differences.

While these negative correlations might well represent "metabolic costs" of

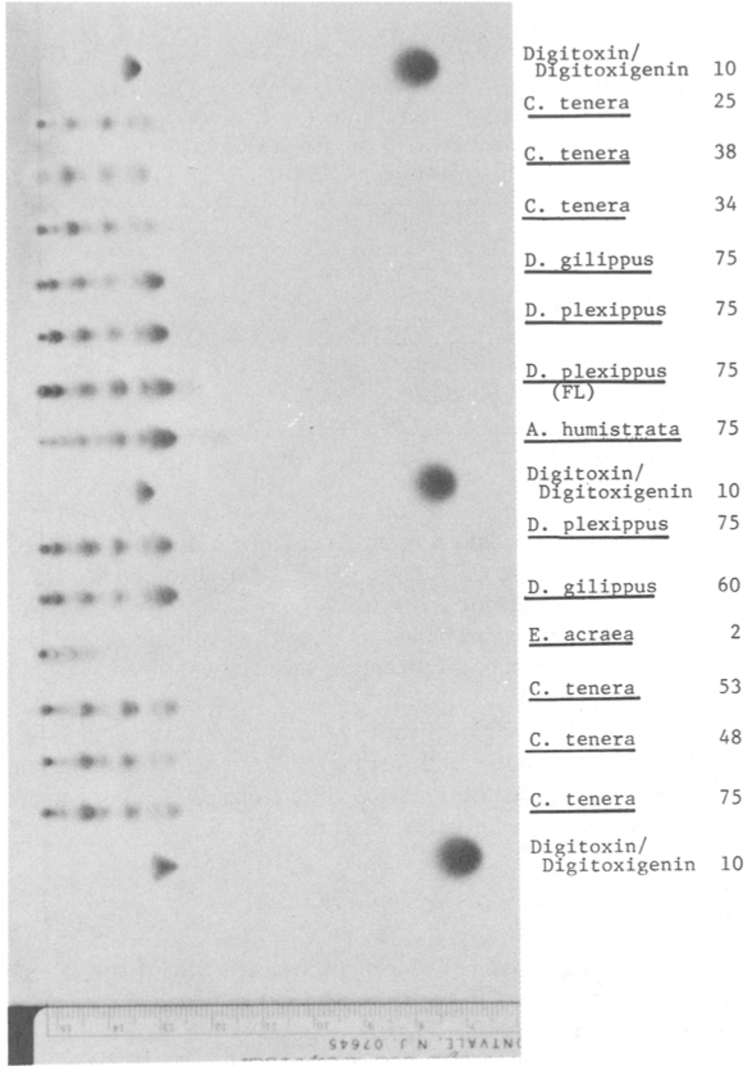


FIG. 4. Chromatograms of adult monarchs and queens reared in the laboratory on *Asclepias humistrata* (developed as in Figure 3). Note that the profiles of the two species are virtually identical, yet differ from those shown in Figure 3. All butterflies were collected as eggs or first instars in the Dominican Republic, except for the monarch labelled "FL," which was collected wild in the egg stage in Florida and shows an identical "fingerprint" pattern to those of Dominican Republic stock. For comparison, extracts of six dogbane tiger moths (*Cycnia tenera*) and one saltmarsh moth (*Estigmene acraea*), reared simultaneously on *A. humistrata,* were spotted on the same silica plate. Also spotted are an ethanolic leaf extract of *A. humistrata* and a 1:1 (v/v) mixture of digitoxin and digitoxigenin. Numbers to the right of each channel indicate the micrograms of cardenolide spotted, calculated prior to lead acetate clean-up.

cardenolide ingestion, as suggested by Brower and Glazier (1975), there is as yet no direct evidence of a causal connection between cardenolide differences and body size differences. Neither Seiber et al. (1980) nor I (Cohen, 1983) found any effect of ingested digitoxin (added to controlled diets) upon the development time, food consumption, or body weight of fourth instar monarch larvae. However, if it is true that the negative correlations do reflect metabolic costs, then we must inquire why such costs should be paid by monarchs and not by queens.

One possibility is that queens are better adapted to cardenolides than are monarchs, due to differences in population structure of these two species. Monarchs are migratory, with most individuals in eastern North America flying to a few restricted areas in Mexico to overwinter (Urquhart and Urquhart, 1977). Mating occurs during the remigration back to North America each spring and most probably results in many matings between individuals which developed as larvae on different species of milkweed, some of which lack cardenolide (see Roeske et al., 1976). Many of these matings would therefore involve butterflies that had not been subjected to selection for allelochemic tolerance. This would tend to recombine any evolving gene complexes for cost-free adaptation to allelochemics. In contrast, queens are fundamentally nonmigratory (Young, 1982), making only limited regional movements (Brower, 1961; Burns, 1983). This greater degree of philopatry should more often result in matings among individuals that fed, as larvae, on the same host plant species. In areas where high-cardenolide plants predominate, this should facilitate the evolution of a more rapid, fine-tuned adaptation to host plant allelochemics.

Two alternative hypotheses to explain the species difference in correlations between body size and cardenolide content are that queens may either effect different metabolic conversions of cardenolides than do monarchs or that they sequester them in less sensitive tissues. Although some data are available on the metabolism (Brower et al., 1982; Marty, 1983) and tissue distribution (Brower, 1984; Brower and Glazier, 1975; Nishio, 1980) of cardenolides in monarchs, no comparable data are yet available for queens. Thus, it is not yet possible to test these alternative hypotheses.

*Sexual Differences in Cardenolide Concentration.* Previous studies of monarchs have found that females have higher cardenolide concentrations than males (Brower and Glazier, 1975; Brower and Moffitt, 1974; Brower et al., 1972). This was again demonstrated here for the laboratory-reared monarchs. Brower and Glazier (1975) suggested that such a dimorphism might reflect relatively stronger selection for chemical defense in females which must spend considerable time exposed to potential enemies while searching for oviposition plants. However, males also incur certain risks in searching for females, and it is not clear how these risks to males are to be compared with risks to females when predicting differences in defensive strategies. In any event, such risks would presumably apply to the congeneric queen as well. The lack of sexual difference in cardenolide content of queens shown here therefore suggests that the "differential risk"

hypothesis is not sufficient to explain the dimorphism previously observed in monarchs.

Another possible explanation is that female monarchs store more cardenolide than males as a means of providing for the defense of their eggs (Brower et al., 1982). Thomashaw (in Brower, 1984) reported that each egg of a female monarch reared on *A. curassavica* contained, on average, 0.97 μg of cardenolide. Since a female may lay from 100 (Erickson, 1973) to 400 (Urquhart, 1960) eggs, these could collectively contain as much as 97–388 μg of cardenolide. Adult monarchs reared on *A. curassavica* contain an average of 670 μg of cardenolide (Roeske et al., 1976). Thus, the amount placed by females into eggs constitutes a substantial proportion (14% to 58%) of this total. One might therefore expect cardenolides to be effective predator or parasite deterrents in monarch eggs, but this has not yet been tested. The lack of sexual difference in cardenolides of queens may suggest that females of this species are not strongly selected to store these compounds for egg defense and leads to the prediction that they should allocate proportionally less cardenolide to eggs than do monarchs. Further work should be directed at this issue.

Interestingly, while laboratory-reared monarch (but, again, not queen) females did have significantly higher cardenolide concentrations than males, this was not observed in field collections from the Miami area. This may suggest that some of the wild-caught females in the Miami sample had already laid some of their cardenolide-rich eggs, thereby reducing their (initially greater) cardenolide loads to a level similar to that of males. (This suggestion is consistent with the laboratory finding of Dixon et al. (1978) that female monarchs that had laid all of their eggs were less emetic when force-fed to pigeons than were freshly eclosed females.) Since females would likely oviposit at varying rates, this should lead to a greater variation in cardenolide concentration of females, relative to males. Indeed, females do tend to have higher variances than males for this trait (see Table 1; $F_{max} = 2.12$, $0.05 < P < 0.10$).

*Cardenolide Variability and its Implications.* The great intra- and interspecific uniformity in qualitative cardenolide profiles of the Miami butterflies (see Figure 3), suggests that a single host plant species had been utilized by larvae of both species but that there is individual variation in storage of certain compounds, especially those of highest $R_f$ value. That these TLC profiles are virtually indistinguishable from those of monarchs reared in the laboratory on *A. curassavica* (Figure 3 inset) strongly suggests that this was the host plant species utilized by both danaid species in the Miami sample (cf. Figure 4 for *A. humistrata*-reared butterflies). This represents the first practical application of the cardenolide "fingerprinting technique" (Brower et al., 1982) to identify the host plants utilized, as larvae, by wild-caught danaid butterflies. It also demonstrates the potential of the technique as an aid in understanding the natural history and migration patterns of danaids. Since the monarchs studied were collected in Miami in December and developed on *A. curassavica* (an introduced milkweed

with a North American distribution restricted to southern states; Woodson, 1954), we may conclude that they were not merely migrant butterflies from northern states that had "become trapped" in peninsular Florida en route to Mexico. Rather, this is strong evidence that monarchs breed in south Florida during the winter months.

A large percentage of each queen sample consisted of butterflies containing no measurable cardenolide (57% in Lake Istokpoga, 17% in Corkscrew Swamp, and 21% in Miami). Since there were no significant sex differences, such intrapopulational variability may instead reflect localized differences in host plant species availability, intraspecific variation in plant cardenolide content (see, e.g., Nelson et al., 1981), or individual butterfly differences in cardenolide sequestration. Whatever its origin, such variability suggests the existence of a cardenolide-based palatability spectrum for queens, similar to that previously described for monarchs (Brower, 1969; Brower et al., 1968; Brower and Moffitt, 1974).

This result has important implications for understanding the southern viceroy's apparent switch from mimicking the monarch (as it does elsewhere in its range), to mimicking the queen in Florida (Brower, 1958a,b). If queens had been found to contain, on average, either more cardenolide than monarchs, or a different and potentially more potent (e.g., more emetic; Brower, 1969) set of cardenolides, then such a mimetic switch might be easily understood. However, the Miami queens clearly contained lower cardenolide concentrations and total amounts than did the sympatric monarchs. Since the two species had virtually identical cardenolide "fingerprints," it cannot be argued that queens stored a more noxious array of cardenolides than did monarchs and were therefore more emetic even at lower concentrations. Thus, when fed on the same plants, monarchs and queens store the same cardenolides but monarchs concentrate these to a greater extent than do queens. This conclusion is further supported by laboratory rearings of the two species on *Asclepias humistrata* (Table 3 and Figure 4). Moreover, Brower et al. (1975) have shown that, in order for an *A. curassavica*-reared monarch (sexes pooled) to be emetic to an 85-g blue jay on 50% of test trials, it must contain at least 76 μg of cardenolide. Of the Miami butterflies analyzed here, 85% of monarchs met this criterion of unpalatability, while only 30% of queens did so. It therefore seems that, at least with respect to cardenolides, queens are poorer models for viceroy mimicry than are monarchs.

Why then should the viceroy have abandoned its usual model in favor of the queen? Since monarchs are migratory, "pulsing" through Florida in large numbers only in the spring and fall (Urquhart and Urquhart, 1976; Brower, Malcolm, and Cockrell, unpublished data), while queens are more sedentary, the latter species would be spatiotemporally more "available" than monarchs to act as models in Florida. A theoretical model developed by Pough et al. (1973) showed that mildly noxious species could serve as suitable models for mimicry if they occurred in sufficient abundance. This may provide the explanation for the switch

in viceroy mimicry. If resident Florida monarch populations have been stable and predictable in their current locations for sufficient time, then reversals in the trend of mimicry might be expected, such that viceroys in those areas should tend to be more "monarch-like" than those elsewhere in the state. A detailed geographic analysis of wing patterns and phenology is needed to test this prediction.

Alternatively, queens might, in fact, be superior models to monarchs, not because of their cardenolide content but, rather, due to sequestered pyrrolizidine alkaloids (PAs) that adults ingest from certain withering plants (Edgar, 1975; Edgar et al., 1979). While both sexes typically store these compounds as adults, male queens employ them further as precursors of their sex pheromone (Meinwald et al., 1969; Pliske and Eisner, 1969). Male and female monarchs are also somewhat attracted to PA sources and may store the alkaloids but males apparently do not use them as pheromone precursors (Edgar et al., 1976). While there is as yet no experimental verification of PA-based defense in danaid butterflies, K.S. Brown (unpublished manuscript) reports that certain neotropical spiders will release ithomiid butterflies from their webs unharmed if they contain PAs. The dependence of male queens (and not monarchs) on PAs for sexual competence suggests that, on average, queens may contain more of these compounds than monarchs, and therefore possibly serve as better models for viceroy mimicry. A comparative study of the PA concentrations of wild-caught monarchs and queens would shed further light on this intriguing problem.

*Adaptation to Plant Allelochemics.* With respect to the issues of herbivore adaptation to allelochemics presented in the Introduction, this study leads to the following provisional conclusions: (1) There is no evidence that the lower cardenolide concentrations sequestered by queens relative to monarchs reflects a poorer underlying tolerance for these allelochemics. On the contrary, since only monarchs demonstrate significant negative correlations between body size and cardenolide concentration, it might be argued that monarchs are less adapted than queens for handling cardenolides. However, a causative connection between cardenolide sequestration and body size has not been established (Seiber et al., 1980; Cohen, 1983). (2) Despite quantitative differences between monarchs and queens in cardenolide storage, both species appear to sequester the same individual cardenolide compounds from their host plants. Indeed, when reared on the milkweed *A. humistrata,* even dogbane tiger moths (*Cycnia tenera;* Arctiidae), also apocynad–asclepiad specialists, produced the same characteristic TLC profile (Figure 4; see also Cohen and Brower, 1983) as did the polyphagous arctiid moth, *Estigmene acraea* (although only in trace amounts; Figure 4). Moreover, Marty (1983) has shown that gut homogenates of both monarch and *E. acraea* larvae are capable of effecting a similar enzymatic transformation of one milkweed cardenolide, uscharidin, to two more polar metabolites (calactin and calotropin). Such similarity among taxonomically disparate Lepidoptera,

whether oligo- or polyphagous, suggests that there may exist only a single *qualitative* route of cardenolide processing in this insect order but that further evolution may involve a *quantitative* increase in tissue cardenolide concentration in accordance with the defensive requirements of each species. However, the basic biochemical processes shared by all these species may represent a common preadaptation to feeding on milkweeds or other cardenolide-containing plants.

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# MEASURING MINIMAL CONCENTRATIONS OF ATTRACTANTS DETECTED BY THE NEMATODE *Panagrellus redivivus*

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**Abstract**—A simple method for the experimental determination of minimal concentrations of attractants detected by the nematode *Panagrellus redivivus* is described. The lowest concentrations of methyl, ethyl, propyl, butyl, and amyl acetate as well as the minimal differences in concentrations of these attractants detectable by *Panagrellus redivivus* are presented.

**Key Words**—Attraction, attractants, assay, *Panagrellus redivivus*, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, amyl acetate, nematodes.

## INTRODUCTION

In one of our previous papers (Balanová et al., 1979) the attraction of *Panagrellus redivivus* to some metabolites of yeasts and fungi and a computerized estimation of concentration gradients of the attractants to which the nematodes are subjected during assay (Balan and Gerber, 1972) were presented. The simulated computation of concentration profiles in the assay assembly indicated that the nematodes reacted to attractants (methyl, ethyl, propyl, butyl, and amyl acetate) in concentrations ranging from $1 \times 10^{-7}$ to $1 \times 10^{-10}$ mol/liter.

We wanted to verify this computerized estimation experimentally, but no suitable method for such work was known. The obvious problem in work of this type is to detect the exact moment at which the random movement of a nematode in an attractant gradient changes to a directed movement towards the attractant and to determine the threshold concentration of the attractant to which the nematode was subjected at that very moment.

A slightly different problem in studying attraction is the determination of the lowest increment of an attractant which can still affect the movement of a



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nematode. This value can be useful in estimating appropriate gradients for effective attraction.

A novel bioassay was developed for the experimental determination of minimal concentrations (or increments) of attractants which nematodes are able to detect.

## METHODS AND MATERIALS

*Procedure.* In the center of a 3-mm layer of plain 3% agar in a 80-mm-diameter Petri dish two 3-mm-thick, 1-cm-diameter 2% agar disks with the attractant (A) and two similar control disks not containing the attractant (C) are positioned alternately and next to each other forming a square (Figure 1).

The needed concentration of the attractant (all are liquids sparingly soluble in water) in agar is obtained by the dilution of the saturated solution of the attractant in water at 20°C and by further dilution (1 : 1) with melted agar cooled to 40°C. After perfect mixing, the agar is poured into a Petri dish and forms a 3-mm layer of 2% agar containing the needed concentration of the attractant. From the solidified agar, disks are cut with a cork borer, and they, along with

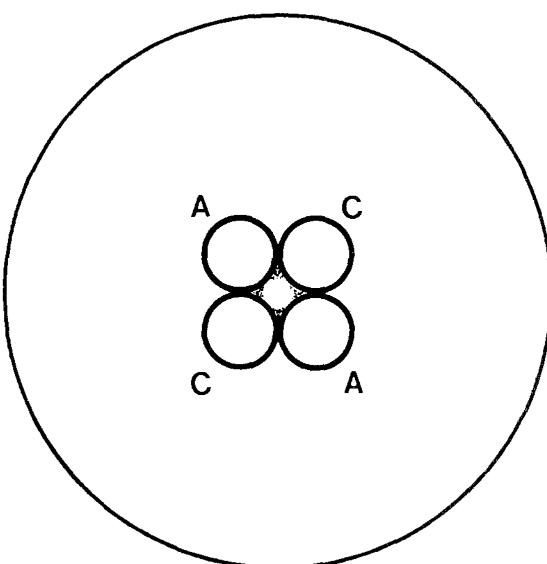


FIG. 1. Experimental arrangement for determining minimal concentrations of attractants detected by the nematode *Panagrellus redivivus.* (For details see "Procedure" in Methods and Materials).

similar control disks, are carefully transferred to the center of the 3% agar layer in the experimental Petri dish.

In this way, a diamond-shaped well is formed into which about 1500 nematodes in a drop of water are pipetted. Capillary forces immediately move the nematodes to the pointed ends of the wedge-like tips of the well. The nematodes are thus gently moved to an ideal "starting position" practically in direct contact with two known concentrations of an attractant in the adjacent walls of each wedge and can move into or under either type of disk.

After 10, 30, and 60 min, the position of the nematodes is checked through the agar with a low-power stereoscopic microscope. Effective concentrations of attractants cause nearly all nematodes to accumulate under and in the disks containing the attractant.

For the determination of the lowest concentration differences which are still detected by the nematodes, the procedure is identical with that described, but two and two disks containing different concentrations of one attractant (and no control disks) are used.

Because of the exceptional ability of the nematodes to detect even very slight differences in concentrations of attractants, the dilution procedure has to be devised very carefully. To avoid dilution errors (which could easily exceed the detection threshold of nematodes for differences in concentrations of attractants), it is of utmost importance to start the last step of dilution from a single stock agar of the higher attractant concentration, dividing it into two portions and further diluting one of them with a small volume (e.g., 1%) of melted 2% agar.

*Nematode. Panagrellus redivivus* (Linn) Goodey 1945 is cultivated according to Balanová et al. (1979). A suspension containing about $3 \times 10^4$ thoroughly washed nematodes was used.

*Attractants.* Analytical grade methyl acetate, ethyl acetate, propyl acetate, butyl acetate, and amyl acetate (Lachema, Brno, Czechoslovakia).

*Agar.* Bacteriological agar No. 1 (Oxoid).

## RESULTS AND DISCUSSION

In Figures 2–5, photographs of agar disks (enlarged by about 4.2 diameters) containing different concentrations of amyl acetate are shown. Ten minutes after the beginning of the experiment nearly all nematodes have accumulated under and in the disks containing $6.24 \times 10^{-7}$ mol of amyl acetate per liter (Figure 2, disks A) whereas in the control disks (C) practically no nematodes can be seen. Similar results can be seen in Figure 3, where a concentration of amyl acetate two orders lower ($6.24 \times 10^{-9}$ mol/liter) was tested. The concentration of $6.24 \times 10^{-10}$ mol/liter (Figure 4) showed no attraction. The nematodes were

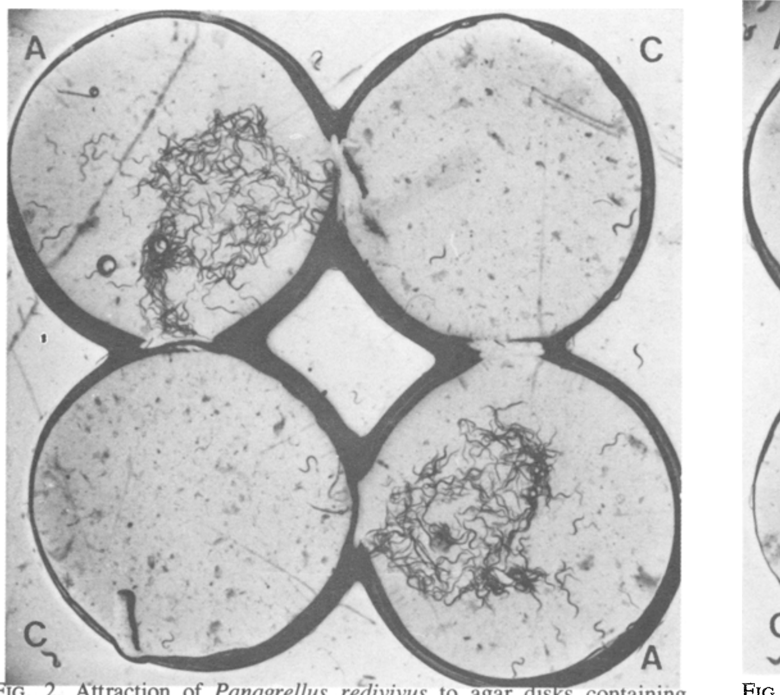


FIG. 2. Attraction of *Panagrellus redivivus* to agar disks containing $6.24 \times 10^{-7}$ mol of amyl acetate per liter (A) 10 min after beginning the experiment; enlarged by about 4.2 diameters. C = control disks.

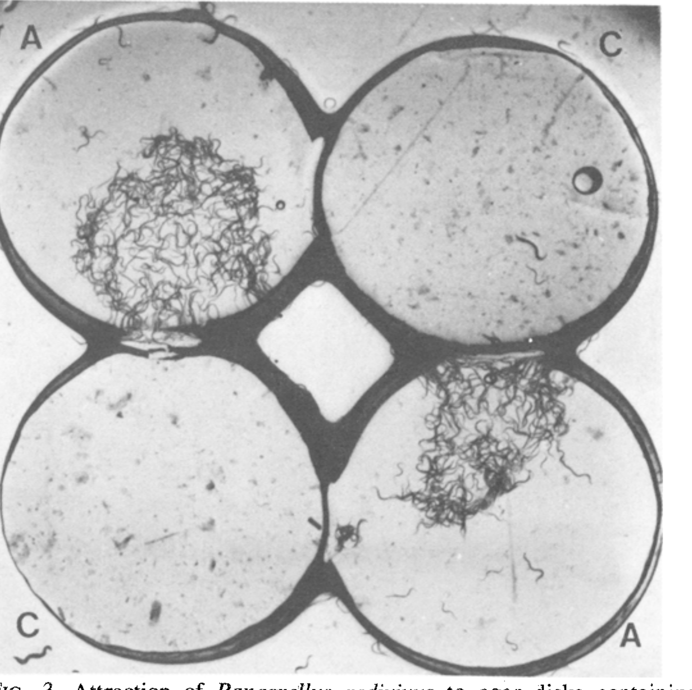


FIG. 3. Attraction of *Panagrellus redivivus* to agar disks containing $6.24 \times 10^{-9}$ mol of amyl acetate per liter (A) 10 min after beginning the experiment; enlarged by about 4.2 diameters. C = control disks.

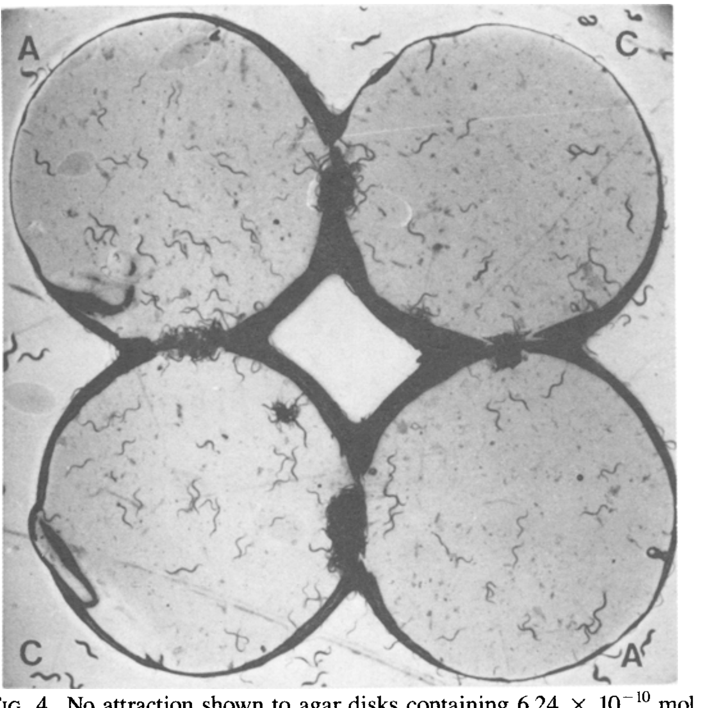


FIG. 4. No attraction shown to agar disks containing $6.24 \times 10^{-10}$ mol of amyl acetate per liter (A). Nematodes are evenly distributed but masses of nonattracted nematodes can be seen at the interfaces between the disks 10 min after beginning the experiment, enlarged by about 4.2 diameters. C = control disks.

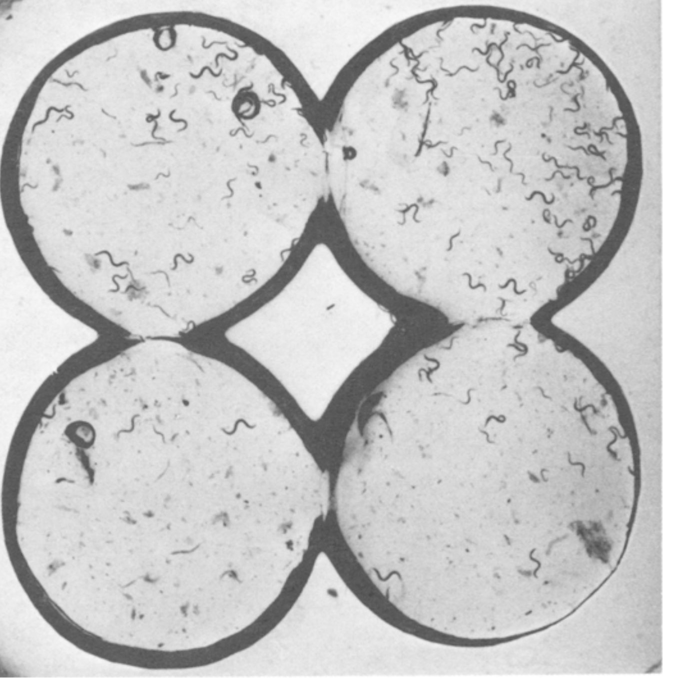

FIG. 5. Control experiment with four disks containing no attractant. No crowding of nematodes at the interfaces between the disks can be observed. Most nematodes have moved out of the well to the perimeter of the Petri dish (not seen in the figure) 10 min after beginning the experiment; enlarged by about 4.2 diameters.

TABLE 1. LOWEST CONCENTRATIONS OF ATTRACTANTS DETECTED BY NEMATODE *Panagrellus redivivus*

| Attractant | Lowest detected concentration in | |
|---|---|---|
| | ppm | mol/liter |
| Methyl acetate | 1.0 | $1.35 \times 10^{-5}$ |
| Ethyl acetate | 0.1 | $1.14 \times 10^{-6}$ |
| Propyl acetate | 0.01 | $9.79 \times 10^{-8}$ |
| Butyl acetate | 0.1 | $8.60 \times 10^{-7}$ |
| Amyl acetate | 0.0009 | $6.24 \times 10^{-9}$ |

evenly distributed, and masses of nematodes can be seen packed at the interfaces between the disks.

The disks of a control experiment are presented in Figure 5. No accumulation of nematodes can be observed at the interfaces of the disks, and the nematodes are evenly distributed but most have moved out of the well to the perimeter of the Petri dish (not seen in the figure).

The lowest effective threshold concentrations of five attractants are presented in Table 1. In evaluating the results presented in the table, one has to keep in mind that the actual concentrations of attractants in the agar disks is evidently even somewhat lower than indicated, as all the tested substances are volatile. Some loss cannot be avoided during the preparation of the disks, and obviously the losses of the lower-molecular-weight attractants (having a lower boiling point) are apt to be somewhat greater than the losses of the attractants with a higher molecular weight.

For determining the lowest concentration differences of these attractants which are detected by *Panagrellus redivivus*, we worked with concentrations two orders higher than the threshold concentrations presented in Table 1. Under these experimental conditions, the nematodes were able to detect a concentration difference of 0.1% in amyl acetate and a concentration difference of 1% in the remaining four attractants.

The established effective threshold concentrations of these attractants are in agreement with the computed estimate which was based on completely different experimental conditions. In the paper by Balanová et al. (1979), we estimated that substances of this type have an effective threshold concentration somewhere between $1 \times 10^{-7}$ and $1 \times 10^{-10}$ mol/liter. Both approaches to this problem seem to be feasible, but the experimental method presented in this paper is much more precise, less tedious, and more direct.

We are convinced that this method could also be used or adapted for other nematodes and/or for the research of nematode-repellent substances.

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# SIMPLE AND ECONOMIC SYNTHESES OF SOME (Z)-7- AND (Z)-9-ALKENYL ACETATES, AND OF (*E*,*Z*)-7,9-DODECADIEN-1-YL ACETATE, THE SEX PHEROMONE OF THE EUROPEAN GRAPEVINE MOTH, USING ALEURITIC ACID AS A COMMON STARTING MATERIAL

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**Abstract**—Short syntheses of (Z)-7-dodecen-1-yl acetate, (Z)-7-tetradecen-1-yl acetate, (Z)-9-dodecen-1-yl acetate, and (Z)-9-tetradecen-1-yl acetate from 7-hydroxyheptanal and 9-oxononanoic acid precursors obtained by oxidative cleavage of easily available aleuritic acid are reported. The key step in these syntheses is a stereoselective Wittig reaction between aldehyde and alkylphosphonium salt. Wittig-Horner type reaction of 7-hydroxyheptanal and diethyl cyanomethylphosphonate gave the $\alpha,\beta$-unsaturated nitrile derivative which after protection of the hydroxyl group was reduced to the corresponding aldehyde. Wittig reaction of the latter, followed by acetylation, completed the synthesis of (*E*,*Z*)-7,9-dodecadien-1-yl acetate, the sex pheromone of the European grapevine moth *Lobesia botrana* Schiff.

**Key Words**—Sex pheromone, synthesis, aleuritic acid, (Z)-7-dodecen-1-yl acetate, (Z)-7-tetradecen-1-yl acetate, (Z)-9-dodecen-1-yl acetate, (Z)-9-tetradecen-1-yl acetate, (*E*,*Z*)-7,9-dodecadien-1-yl acetate, European grapevine moth, *Lobesia botrana*, Wittig reaction, $\alpha,\beta$-unsaturated aldehyde.

## INTRODUCTION

The sex pheromones of Lepidoptera are usually unsaturated straight-chain alcohols, acetates, aldehydes, or hydrocarbons (Inscoe, 1982). The efficient syn-



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thesis of these seemingly simple substances requires stereoselective construction of the olefinic bond having the proper configuration (*E* or *Z*) from suitably selected starting materials (see the reviews of Henrick, 1977; Mori, 1979). In a given case, economic considerations determine the choice of alternative methods. Furthermore, an economical process for their large-scale manufacture generally requires starting material which is readily accessible from natural sources.

Shellac, a commercially available natural resin is produced from a protective resinous incrustation secreted by the insect *Laccifer lacca* Kerr (Lacciferdiae Cockerell), which is indigenous to southeast Asia. Aleuritic acid I [(±)-threo-9,10,16-trihydroxyhexadecanoic acid), the main consituent of shellac, can be readily obtained by saponifying dewaxed lac (Martin, 1982). Constant yields of nearly 43% of aleuritic acid from decolorized and dewaxed shellac have been reported (Gidvani, 1944).

The ready availability of this versatile multifunctional acid has prompted several research groups to use it for the synthesis of various biologically active compounds, such as prostanoids (Burton et al., 1976; Reuter and Salomon, 1978), fluoro-containing termiticides (Prestwich et al., 1981), and an analog of the gypsy moth pheromone (Subramanian et al., 1982). Recently, Muchowski and Venuti (1981) have used 7-hydroxyheptanal (II) obtained by cleavage of I in their interesting synthesis of the sex pheromone of the pink bollworm moth (gossyplure). Most recently, Chattopadhyay et al. (1983) reported the synthesis of queen bee and cabbage looper pheromones from aleuritic acid.

In this paper we describe the use of aleuritic acid I [(±)-threo-9,10,16-trihydroxyhexadecanoic acid) as a common starting material for the syntheses of (Z)-7-dodecen-l-yl acetate V, (Z)-7-tetradecen-l-yl acetate VI, (Z)-9-dodecen-l-yl acetate XII, and (Z)-9-tetradecen-l-yl acetate IX, which frequently occur in the pheromone blend of moth and butterflies (Insocoe, 1982). A short synthesis of (*E*,*Z*)-7,9-dodecadien-l-yl acetate XVII, the sex pheromone of the European grapevine moth *Lobesia botrana* Schiff. (Buser et al., 1974; Roelofs et al., 1973) taking advantage of an improved preparation of (*E*)-9-hydroxy-2-nonenal XV derivative is also presented.

## METHODS AND MATERIALS

IR spectra were obtained with a Pye Unicam SP-3-200 spectrometer. [$^{1}$H]NMR data were determined at 60 MHz on a Hitachi Perkin Elmer R-24/A spectrometer. [$^{13}$C]NMR spectra were obtained by broad-band decoupled proton noise decoupled operation at 62.9 MHz using a Bruker AM-250 spectrometer. Chemical shifts were measured against TMS as an internal standard. Following abbrevations are used: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br). Mass spectra were determined on a JEOL-2OK and JMS-O1SG-2 combined GC-MS system. (Ionizing energy 74 eV, accelerating voltage 10 kV, ionizing current 200 $\mu$A).

The isomeric purity of monoenic products was greater than 98% as determined from their [$^{13}$C]NMR and IR spectra.

Solvents were purified and dried in a conventional manner. The aleuritic acid was purchased from Fluka AG.

*Cleavage of Aleuritic Acid.* One hundred fifty-one grams (0.494 mol) of aleuritic acid I was added to a solution of 19.8 g (0.494 mol) of NaOH in 600 ml of water. After vigorous stirring for 15 min, 600 ml of $CHCl_3$ was added, then 127 g (0.594 mol) of $NaIO_4$ was added gradually, and the mixture was cooled to 20° during 30 min. The precipitate was filtered off, the filter cake was washed with 80 ml $CHCl_3$. The aqueous phase of the filtrate was extracted with 80 ml of $CHCl_3$ and then set aside. The organic extract was stirred for 1 hr with a mixture of 240 ml of saturated $NaHCO_3$ and 100 ml of saturated $Na_2CO_3$ solutions, and then the aqueous carbonate phase was separated and extracted with 2 × 100 ml of $CHCl_3$. The combined organic extracts were washed with 250 ml of water, 150 ml of brine, dried over $MgSO_4$, and the solvent was removed in vacuo to afford 54.2 g (89% yield) of 7-hydroxyheptanal (II), which solidified on standing (mp 65–67°). IR ($CCl_4$): 3420, 2940, 2860, 2720, 1720, and 870 $cm^{-1}$, [$^1$H]NMR($CDCl^3$): $\delta$ 1.1–1.9 (8H, m, $CH_2$), 2.35 (2H, t, $J$ = 6 Hz, $CH_2$), 2.45 (1H, s, OH), 3.55 (2H, t, $J$ = 6 Hz, $OCH_2$), and 9.52 (1H, t, $J$ = 2 Hz, CHO).

Two hundred fifty milliliters of $CHCl_3$ was added to the aqueous phase obtained from the cleavage. The resultant mixture was acidified with 10% HCl at 5° (pH = 2), the aqueous phase was separated and extracted with 3 × 100 ml of $CHCl_3$, the combined organic extracts were dried over $MgSO_4$, approx. 0.1 g of hydroquinone was added, and the solvent was removed in vacuo to give 75.4 g (89% yield) of crude 9-oxononanoic acid (III), as a viscous oil. IR(neat): 3700–2500, 2930, 2860, 2720, 1720, 1710 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 1.1–1.8 (10H, m, $CH_2$), 2.3 (4H, t, $J$ = 6 Hz, $CH_2$), 9.55 (1H, t, $J$ = 2 Hz, CHO), 9.85 (1H, bs, COOH).

*7-Acetoxyheptanal (IV).* Twenty-two milliliters (0.22 mol) of acetic anhydride was added dropwise during 10 min to a solution of 15 g (0.115 mol) of aldehyde II, 80 ml of anhydrous benzene and 22 ml of anhydrous pyridine at 10°. After stirring 5 hr at room temperature, the mixture was poured onto crushed ice, the aqueous phase was extracted with 2 × 25 ml of hexane, the combined organic solutions were washed with 10% HCl solution, 5% $Na_2CO_3$ solution, and brine, and dried over $MgSO_4$. Evaporation of the solvent and distillation in vacuo afforded 16.0 g (80% yield) of pure aldehyde IV (bp 78–81°/0.8 mm Hg). IR(neat): 2720, 1740, 1240, and 1035 $cm^{-1}$, [$^1$H]NMR($CDCl_3$): $\delta$ 1.0–1.9 (8H, m, $CH_2$), 1.95 (3H, s, $CH_3$), 2.35 (2H, T, $J$ = 6 HZ, $CH_2$), 3.9 (2H, T, $J$ = 6 HZ, $OCH_2$), 9.5 (1H, t, $J$ = 2 Hz, CHO).

(Z)-*7-Dodecen-l-yl Acetate (V).* To a stirred solution of sodium methylsulfinyl methanide (prepared from 0.083 mol of NaH suspension and 40 ml of anhydrous DMSO), 32.5 g (0.078 mol) of pentyltriphenylphosphonium bromide in 70 ml anhydrous DMSO was added during 15 min at 15–20° under dry argon

atmosphere. After stirring for 30 min at room temperature, the solution was cooled and 9.0 g (0.052 mol) of aldehyde IV in 5 ml anhydrous DMSO was added dropwise during 1 min at 15°. The mixture was stirred for 10 hr at room temperature, poured onto ice-water, and extracted with 4 × 100 ml of hexane. The combined organic extracts were washed with water and brine, dried over $MgSO_4$, and concentrated to one third of the volume. The residue was held for 10 hr at −10°, and the precipitate was filtered off.

After evaporation of the solvent, the crude product was distilled to afford 6.9 g (59% yield) of pure acetate V (bp 102–104°/0.05 mm Hg). IR(neat): 1740, 1460, 1360, 1235, 1040, and 670 $cm^{-1}$; [$^{1}H$]NMR($CDCl_3$): $\delta$ 0.95 (3H, t, $J$ = 6 Hz, $CH_3$), 1.1–1.5 (2H, m, $CH_2$), 1.9–2.1 (7H, s+m, $CH_3$ and $CH_2$), 3.95 (2H, t, $J$ = 6 Hz, $OCH_2$), 5.2 (2H, m, CH=CH). [$^{13}C$]NMR($CDCl_3$): $\delta$ 13.9 ($CH_3$), 20.8 ($CH_3$), 22.4, 26.0, 27.1, 27.3, 28.9, 29.0, 29.8, 32.1, 64.9 ($9CH_2$), 129.8 and 130.2 (4CH=), 170.8 (C=O). MS; $M^+$ 226 (<1), *m/e* 166 (35), 123 (12), 110 (33), 109 (27), 96 (69), 95 (42), 82 (89), 81 (65), 68 (46), 67 (80), 43 (100), 41 (67).

Analysis: calc. for $C_{14}H_{26}O_2$: H 11.58, C 74.28; found: H 11.82, C 73.91%.

*(Z)-7-Tetradecen-l-yl Acetate (VI).* To an ice-cooled and stirred suspension of 10.0 g (0.0227 mol) of heptyltriphenylphosphonium bromide in 50 ml of anhydrous THF, 2.5 g (0.0227 mol) of potassium *tert*-butoxide was added at once and the mixture was stirred for 30 min at room temperature under argon atmosphere. The resulting solution was cooled to −78° and 3.4 g (0.02 mol) of aldehyde IV in 5 ml of dry THF was added dropwise during 2 min. After stirring for 1 hr at −78°, 1 hr at 0°, and 1 hr at room temperature, the solvent was evaporated at reduced pressure, the residue was stirred vigorously with 30 ml of cold water and 30 ml of hexane, the aqueous layer was separated and extracted 2 × 20 ml of hexane, the combined organic extracts were washed with water and brine, dried over $MgSO_4$, and concentrated to one third of its volume. The residue was held for 10 hr at −15°, and the precipitate was filtered off. The filtrate was concentrated, and the residue was distilled to afford 2.8 g (56% yield) of acetate VI (bp 105–110°/0.1 mm Hg).

IR(neat): 1745, 1650, 1470, 1365, 1235, and 1035 $cm^{-1}$, [$^{1}H$]NMR($CDCl_3$): $\delta$ 0.9 (3H, t, $J$ = 6 Hz, $CH_3$), 1.1–1.6 (16H, m, $CH_2$), 1.98 (3H, s, $CH_3$), 1.8–2.2 (4H, m, $CH_2$), 4.0 (2H, t, $J$ = 6 Hz, $OCH_2$), 5.3 (2H, m, HC=CH). [$^{13}C$]NMR($CDCl_3$): $\delta$ 14.1 and 20.7 ($2CH_3$), 22.8, 26.1, 27.3, 27.4, 28.9, 29.1, 29.8, 29.9, 32.0, and 64.5 ($10CH_2$), 129.7 and 130.2 (HC=CH), 170.4 (C=O).

Analysis: calc. for $C_{16}H_{30}O_2$:H 11.89, C 75.53; found: H 11.96, C 75.11%.

*(Z)-9-Tetradecenoic Acid (VII).* Six and three quarter grams (0.06 mol) of potassium *tert*-butoxide was added to an ice-cooled suspension of 24.8 g (0.06 mol) of pentyltriphenylphosphonium bromide and 250 ml of dry THF under argon atmosphere. The resulting bright red solution was stirred for 30 min at room temperature, then cooled to −78° and 5.1 g (0.03 mol) of acid–aldehyde

III in 15 ml of dry THF was added dropwise during 5 min. The mixture was stirred for 45 min at −78°, for 1 hr at 0°, and for 30 min at room temperature, then diluted with 50 ml of water, and the solvent was evaporated. The residue was taken up in 50 ml of 5% $Na_2CO_3$ solution, extracted with 2 × 100 ml of ether, the ice-cooled aqueous phase was acidified with HCl solution, and extracted with 4 × 80 ml of ether. These ethereal extracts were combined, dried over $MgSO_4$, and concentrated to give 5.1 g (76% yield) of crude acid VII.

IR(neat): 3550–2500, 1710, 1440, 1410, 1260, 1100, and 720 $cm^{-1}$. [$^1$H]NMR($CDCl_3$): $\delta$ 0.85 (3H, t, $J$ = 6 Hz, $CH_3$), 1.1–1.6 (14H, m, $CH_2$), 1.9 (4H, m, $CH_2$), 2.25 (2H, t, $J$ = 6 Hz, $CH_2$ C=O), 5.1 (2H, m, HC=CH), 8.6 (1H, bs, $CO_2H$).

(Z)-*9-Tetradecen-l-ol (VIII).* Five grams (0.022 mol) of acid VII in 15 ml anhydrous ether was added dropwise to a stirred suspension of 1.3 g (0.034 mol) of $LiAlH_4$ in 40 ml of anhydrous ether. After refluxing for 2 hr, the mixture was cooled and 30 ml of water followed by 30 ml of 4 N HCl solution was added, and the organic layer was separated. The aqueous layer was extracted with 2 × 30 ml of ether, and the combined ethereal solutions were washed with water, saturated $NaHCO_3$ solution, and brine, dried, and evaporated to afford 3.2 g (68% yield) of crude alcohol VIII.

IR(neat): 3350, 3000, 1640, 1050, 720 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 0.9 (3H, t, $J$ = 6 Hz, $CH_3$), 1.1–1.5 (16H, m, $CH_2$), 1.95 (4H, br s, $CH_2$), 2.1 (1H, s, OH), 3.56 (2H, t, $J$ = 6 Hz, $OCH_2$), 5.15 (2H, m, HC=CH).

(Z)-*9-Tetradecen-l-yl Acetate (IX).* A solution of 2.0 g (0.0094 mol) of alcohol VIII, 5 ml of dry pyridine, and 2 ml of acetic anhydride was stirred for 5 hr at room temperature; then it was poured onto a mixture of 20 g of ice and 10 ml of 4 N HCl solution. After extraction with 3 × 30 ml of hexane, the organic solution was washed with water, saturated $NaHCO_3$ solution, and brine, then dried and evaporated. The crude product was purified by column chromatography using hexane-ethyl acetate 10:0.5 as eluant to give 1.2 g (50% yield) of acetate IX.

IR(neat): 1740, 1235, and 1035 $cm^{-1}$, [$^1$H]NMR($CDCl_3$): $\delta$ 0.9 (3H, t, $J$ = 6 Hz, $CH_3$), 1.25 (16H, m, $CH_2$), 1.95 (7H, br s, $CH_2$), 3.95 (2H, t, $J$ = 6 Hz, $OCH_2$), 5.15 (2H, m, HC=CH). [$^{13}$C]NMR($CDCl_3$): $\delta$ 13.9 ($CH_3$), 22.4, 26.1, 27.1, 27.4, 28.9, 29.3, 29.5, 30.1, 32.2, 64.7 (10$CH_2$), 129.9 and 130.1 (HC=CH), 170.8 (C=O). MS: $M^+$ 254 (4), *m/e* 194 (35), 183 (20), 165 (8), 152 (8), 151 (6), 138 (15), 137 (13), 124 (22), 122 (17), 110 (29), 108 (17), 97 (23), 96 (94), 82 (100), 67 (69), 55 (70), 43 (65).

Analysis: calc. for $C_{16}H_{30}O_2$: H 11.89, C 75.53; found: H 12.02, C 75.31%.

(Z)-*9-Dodecenoic Acid (X).* This compound was prepared analogously to VII from 67.7 g (0.153 mol) of triphenylpropylphosphonium iodide, 21.1 g (0.187 mol) of potassium *tert*-butoxide, and 19.0 g (0.11 mol) of acid aldehyde III in THF (85% yield).

IR(neat): 3600–2400, 1930, 1710, 1410, and 720 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 0.9 (3H, t, $J$ = 6 Hz, $CH_3$), 1.1–1.6 (10H, m $CH_2$), 1.9 (4H, m, $CH_2$), 2.3 (2H, br s, $CH_2$), 5.15 (2H, m, HC=CH), 8.6 (1H, bs, $CO_2H$).

(Z)-*9-Dodecen-l-ol (XI)*. This compound was prepared from 18.9 g (0.093 mol) of acid X and 5.5 g (0.144 mol ) of $LiAlH_4$ in ether (55% yield) (bp 90–93°/0.25 mm Hg.) IR(neat): 3340, 1055 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 0.9 (3H, t, $j$ = 6 Hz, $CH_3$), 1.1–1.4 (12H, br s, $CH_2$), 2.0 (1H, s, OH), 2.05 (4H, m, $CH_2$), 3.5 (2H, m, $CH_2$), 5.15 (2H, m, HC=CH).

(Z)-*9-Dodecen-l-yl Acetate (XII)*. This compound was prepared analogously to VIII from 3.8 (0.02 mol) of alcohol XI, 4.5 ml of acetic anhydride, and 10 ml of dry pyridine (73% yield). IR(neat): 3000, 1745, 1470, 1390, 1245, 1040, and 680 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 0.95 (3H, t, $J$ = 6 Hz), 1.1–1.7 (15H, m, $CH_2$), 1.95 (4H, m, $CH_2$), 3.95 (2H, t, $J$ = 6 Hz, $OCH_2$), 5.15 (2H, m, HC=CH). [$^{13}$C]NMR($CDCl_3$): $\delta$ 14.3 ($CH_3$), 20.6 ($CH_2$), 20.8 ($CH_3$), 26.1, 27.2, 28.9, 29.3, 29.5, 64.6 (7$CH_2$), 129.4 and 131.7 (HC=CH), 170.8 (C=O). MS: $M^+$ 226 (<1), *m/e* 166 (20), 137 (6), 124 (9), 110 (21), 96 (40), 95 (37), 82 (87), 81 (54), 69 (48), 68 (100), 55 (77), 44 (92).

Analysis: calc. for $C_{14}H_{26}O_2$: H 11.58, C 74.28; found: H 11.80, C 74.01%.

(E)-*9-Hydroxy-2-nonenenitrile (XIII)*. A solution of 86.7 g (0.489 mol) of diethyl cyanomethyl phosphonate (Teissiere et al., 1964) in 150 ml of anhydrous ether was slowly added to an ice-cooled and stirred mixture of 14.8 g (0.493 mol) of NaH (80% dispersion in oil) in 600 ml of anhydrous ether and 200 ml of anhydrous THF. After stirring for 1 hr at room temperature, 64.0 g (0.492 mol) of aldehyde II in 200 ml of anhydrous THF was added during 15 min and below 15°. Then the mixture was vigorously stirred for 2 hr at room temperature. The liquid phase was then decanted, and the gummy phase was washed with ether. The combined organic phases were washed successively with 5% $Na_2CO_3$ solution, water, and brine, and dried over $MgSO_4$. The solvent was evaporated and the residue distilled to give 44.9 g (60% yield) of pure nitrile XIII, as an oil (bp 103–107°/0.1 mm Hg).

IR(neat): 3300, 3080, 3060, 2950, 2870, 2230, 1640, 1465, 1060, 970, and 745 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 1.35 (8H, bs, $CH_2$), 2.3 (2H, m, $CH_2$), 2.9 (1H, s, OH), 3.45 (2H, T, $J$ = 6 Hz, $CH_2$), 5.2 (1H, d+d, $j_{2,4}$ = 2 Hz, $J_{2,3}$ = 16 Hz, CH=), 6.55 (1H, t+d, $J_{2,3}$ = 16 Hz, $J_{3,4}$ = 6 Hz, CH=).

(E)-*9-(1-Ethoxyethoxy)-2-nonenenitrile (XIV)*. *p*-Toluenesulfonic acid (0.05 g) was added to a solution of 40 ml (approx. 0.42 mol) of ethylvinyl ether and 38.8 g (0.253 mol) of nitrile XIII in 150 ml of anhydrous $CH_2Cl_2$ at 18°, and the solution was stirred for 2 hr at room temperature. Then 0.3 g of anhydrous $Na_2CO_3$ and 1 g of $MgSO_4$ were added and, after filtration, the solvent was evaporated invacuo at 30° to give 56.0 g (98% yield) of crude product XIV.

IR(neat): 2980, 2940, 2860, 2230, 1640, 1455, 1380, 1140, 1100, 1090, 1065, and 970 $cm^{-1}$, [$^1$H]NMR($CDCl_3$): $\delta$ 1.0–1.8 (14H, m, $CH_2$ and $CH_3$),

2.35 (2H, m, $CH_2$), 3.35 (4H, m, $OCH_2$), 4.45 (1H, 1, $J$ = 6 Hz, OCHO), 5.1 (1H, d, $J_{2,3}$ = 16 Hz, CH=), 6.45 (1H, d+t, $J_{2,3}$ = 16 Hz, $J_{3,4}$ = 6 Hz, CH=).

(E)-*9-(1-Ethoxyethoxy)-2-nonenal (XV).* Ninety-five milliliters (0.53 mol) of diisobutylaluminium hydride (FLUKA) was added during 45 min to a well-stirred solution of 98.5 g (0.437 mol) of nitrile XIV in 750 ml of anhydrous hexane at −10° under an argon atmosphere. After stirring 3 hr at −10° and 1 hr at 0°, the mixture was cooled to −15° and 10 ml of isopropanol followed by 80 ml of water was added dropwise. The resultant mixture was poured onto a stirred mixture of 500 g of ice and 150 ml of acetic acid. After 10 min of vigorous stirring, the aqueous phase was separated and extracted with 2 × 150 ml of hexane. The combined organic extracts were washed successively with 150–150 ml of 1 N HCl solution, water, saturated $NaHCO_3$ solution, and brine, and dried over $MgSO_4$. Removal of the solvent in vacuo afforded 50.0 g (50% yield) of crude unsaturated aldehyde XV.

IR(neat): 2990, 2940, 2870, 2740, 1700, 1645, 1460, 1450, 1380, 1140, 1110, 1065, 980, and 950 $cm^{-1}$; [$^1$H]NMR($CDCl_3$): $\delta$ 1.0–2.0 (14H, m, $CH_2$ and $CH_3$), 2.2 (2H, m, $CH_2$), 3.3 (4H, m, $OCH_2$), 4.35 (1H, q, $J$ = 6 Hz, OCHO), 5.65 (1H, d+d, $J_{2,3}$ = 16 Hz, $J_{1,2}$ = 8 Hz, CH=), 6.35 (1H, d+t, $J_{2,3}$ = 16 Hz, $J_{3,4}$ = 6 Hz, CH=), 9.2 (1H, d, $J_{1,2}$ = 8 Hz, CHO).

*(E,Z)-7,9-Dodecadien-1-yl Acetate (XVII).* A solution of sodium methylsulfinyl methanide was prepared from 3.3 g (0.11 mol) of NaH (80% dispersion in oil) and 50 ml of anhydrous DMSO. To this solution 45.3 g (0.105 mol) of propyltriphenylphosphonium iodide in 160 ml of anhydrous DMSO was added dropwise at 15°. After stirring for 45 min at room temperature, the mixture was cooled to 10°, and 16.1 g (0.071 mol) of aldehyde XV in 23 ml of anhydrous DMSO was added during 15 min. The reaction mixture was stirred for 4 hr at room temperature, diluted with 150 ml of hexane, and poured onto 1200 g of ice-cold water. The precipitate was filtered off, the filtrate was separated, and the aqueous phase was extracted with 4 × 150 ml of hexane. The combined organic solutions were washed with water and brine, dried over $MgSO_4$, and evaporated to dryness to give 17.1 g of crude diene XVI. The solution of 15.0 g (approx. 0.06 mol) of this diene in 55 ml of acetic acid and 28 ml of acetic anhydride was stirred at 70–75° for 18 hr under argon atmosphere, then the cold mixture was poured onto 250 g of ice-cold water and extracted with 4 × 70 ml of hexane. The combined organic extracts were washed with dilute $Na_2CO_3$ solution, water, and brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed through silica gel. Elution with hexane–ethyl acetate (10:0.7) and distillation of the product gave 6.6 g (40% yield) of dienyl acetate XVII (bp 80–85°/0.1 mm Hg). GLC (2 m × 2.4 mm, 10% Carbowax 20 M on Supelcoport 100–200 mesh) and [$^{13}$C]NMR analyses showed that the ratio of the 7*E*,9*Z* (XVII) and 7*E*,9*E* isomers (XVIII) is 4:1.

IR (neat): 1730, 1640, 1460, 1440, 1380, 1360, 1230, 1030, 980, and 940

$cm^{-1}$; [$^1$H]NMR($CDCl_3$): δ 0.99 (3H, t, $J$ = 7.5 Hz, $CH_3$), 1.35 (8H, m, $CH_2$), 2.04 (3H, s, $COCH_3$), 2.15 (4H, m, $CH_2$), 4.05 (2H, t, $J$ = 6.5 Hz, $OCH_2$), 5.32 (1H, d+t, $J_{6,7}$ = 8 Hz, $J_{7,8}$ = 11 Hz, CH=), 5.60 (1H, d+t, $J_{9,10}$ = 14 Hz, $J_{10,11}$ = 7 Hz, CH=), 5.85 (1H, d+d, $J_{7,8}$ = 11 Hz, $J_{8,9}$ = 10.5 Hz, CH=), 6.20 (1H, m, CH=). [$^{13}$C]NMR($CDCl_3$): δ 14.3 ($CH_3$), 20.8 ($CH_2$), 21.1 ($CH_3$), 26.0, 28.9, 29.4, 29.9, 32.8, 64.6 ($6CH_2$), 126.0, 128.3, 131.7 and 134.2 (4CH=), 170.7 (C=O). MS: $M^+$ 224 (28), *m/e* 164 (12), 163 (4), 149 (3), 136 (7), 135 (16), 123 (3), 122 (8), 121 (23), 109 (6), 108 (18), 107 (16), 105 (3), 97 (3), 96 (20), 95 (45), 94 (24), 93 (36), 91 (13), 83 (8), 82 (44), 81 (36), 80 (26), 79 (67), 77 (19), 67 (100), 66 (8), 61 (7), and 55 (39).

Analysis: calc. for $C_{14}H_{24}O_2$: H 10.78, C 74.95; found: H 10.88, C 74.82.

## RESULTS AND DISCUSSION

For the cleavage of aleuritic acid (Figure 1), Reuter and Salomon used potassium periodate in the presence of $H_2SO_4$. In our hands, the acid-sensitive aldehyde products partially trimerized under these conditions. Therefore we elaborated an improved method of the oxidative cleavage of aleuritic acid. By carrying out the reaction of sodium periodate with the sodium salt of aleuritic acid in a mixture of $CHCl_3$ and $H_2O$, trimerization was not detected, and the reaction yielded a readily separated mixture of products II and III in high yield.

Our synthetic approach to the (Z)-7-alkenyl acetates (Figure 2, V and VI) was based on the Wittig reaction of suitably protected 7-hydroxyheptanal. Thus, acetylation of II with acetic anhydride in pyridine gave 7-acetoxyheptanal (IV) which was reacted with the yield generated from pentyltriphenylphosphonium bromide by sodium methylsulfinylmethanide. The coupling reaction proceeded

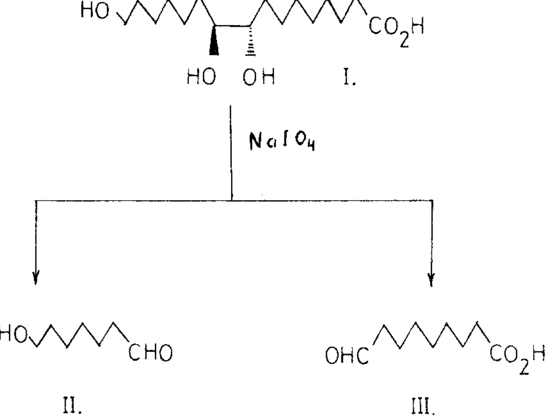


FIG. 1. Cleavage of aleuritic acid.

FIG. 2. Synthesis of (Z)-7-alkenyl acetates.

smoothly in DMSO and afforded (Z)-7-dodecen-1-yl acetate (V) in 47% overall yield. In a similar experiment, treatment of 7-acetoxyheptanal with yield derived from heptyltriphenylphosphonium bromide in anhydrous THF, in the presence of *t*-BuOK afforded (Z)-7-tetradecen-1-yl acetate in 45% overall yield.

The (Z)-9-alkenyl acetates (Figure 3, IX and XII) were prepared from 9-oxononanoic acid (III). Coupling reaction of III with an excess of pentylidenetriphenylphosphorane in THF gave (Z)-9-tetradecenoic acid (VII) in high yield (76%). $LiAlH_4$ reduction in $Et_2O$ followed by acetylation of the intermediate alkenol VIII furnished (Z)-9-tetradecen-1-yl acetate (IX) in 34% overall yield.

In an analogous fashion, (Z)-9-dodecen-1-yl acetate (XII) was synthesized, starting from III and proceeding via intermediates X and XI, in 34% overall yield.

The hydroxyaldehyde II was also a logical starting material for the stereoselective synthesis of the female sex pheromone of the European grapevine moth (XVII). Previous syntheses of (*E,Z*)-7,9-dodecadien-1-yl acetate (XVIII) have been based on the stereoselective Wittig reaction of the $\alpha,\beta$-unsaturated aldehyde or its derivatives, stereoselective reduction of enyne derivatives, or metal-catalyzed cross-coupling of alkenyl derivatives (Henrick, 1977; Rossi et al., 1981; Ideses et al., 1982 and references therein; Szántay et al., 1982; and Cassani et al., 1983).

We have carried out a four-step synthesis of pheromone XVII using a modified Wittig-olefination as the key step (Figure 4). Reaction of the hydroxyalde-

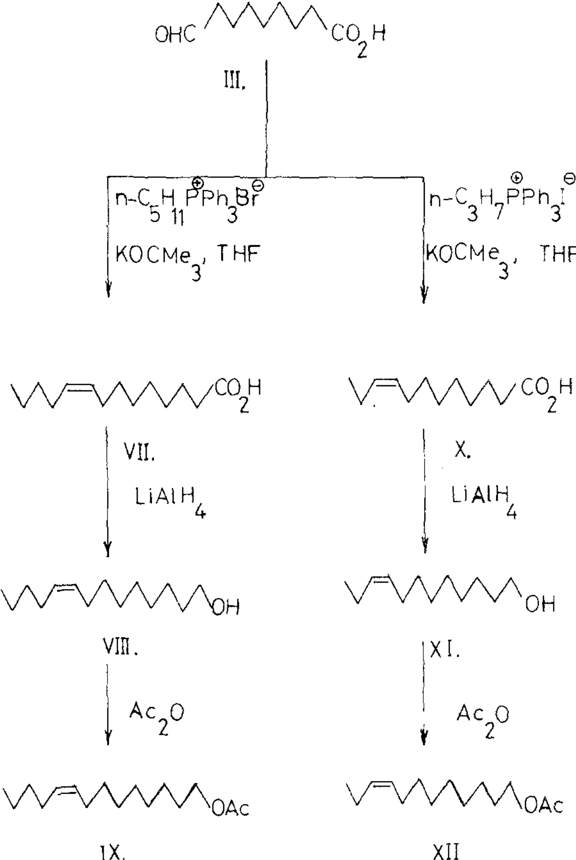


FIG. 3. Synthesis of (*Z*)-9-alkenyl acetates.

hyde II with the ylide derived from diethyl cyanomethylphosphonate and NaH gave $\alpha,\beta$-unsaturated nitrile XIII with the desired *E* bond geometry. The hydroxy group of this nitrile was protected by treating XIII with ethyl vinyl ether and a catalytic amount of *p*-toluenesulfonic acid to give XIV. This was reduced with diisobutylaluminium hydride in anhydrous hexane to afford stereoisomerically pure $\alpha,\beta$-unsaturated aldehyde XV.

Coupling reaction of the latter with the ylide generated from propyltriphenylphosphonium iodide by dimsyl sodium, followed by acetylation of the resulting crude dienol XVI, gave a 4:1 mixture of 7*E*,9*Z* and 7*E*,9*E* isomers of 7,9-dodecadiene-1-yl acetate (XVII and XVIII, respectively). The overall yield of dienyl acetate from II by this four-step synthesis was 14.4%.

Although the 7*E*,9*E* isomer (XVIII) can be removed by reacting the crude product with tetracyanoethylene (Ideses et al., 1982), it is unnecessary because,

$(EtO)_2P(O)CH_2CN$ / NaH
II → XIII
$H^{\oplus}$ → XIV
DIBAL-H → XV
$n\text{-}C_3H_7P^{\oplus}Ph_3I^{\ominus}$ / NaH, DMSO → XVI
$Ac_2O$ / AcOH → XVII + XVIII
4 : 1

FIG. 4. Synthesis of the sex pheromone of the European grapevine moth.

as has been shown (Ideses et al., 1982; Voigt and Ujváry, 1984), 15–20% of *7E,9E* isomeric impurity does not influence the attractivity of the pheromone (XVII) in field experiments.

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# INTENSIFICATION AND PROLONGATION OF HOST SEARCHING IN *Leptopilina heterotoma* (THOMSON) (HYMENOPTERA: EUCOILIDAE) THROUGH A KAIROMONE PRODUCED BY *Drosophila melanogaster*

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**Abstract**—A chemical cue (kairomone) of the host, larvae of *Drosophila*, was found to influence patch-time allocation of the parasite *Leptopilina heterotoma*. This kairomone is soluble in water and chloroform. The kairomone was purified using thin-layer chromatography. The concentration of the kairomone increases with an increasing number of hosts. The parasites may use presence and concentration of the kairomone as cues to determine patch-time allocation.

**Key Words**—*Leptopilina heterotoma*, Hymenoptera, Eucoilidae, *Drosophila melanogaster*, Diptera, Drosophilidae, host searching, kairomone, concentration effect.

## INTRODUCTION

The process leading to parasitization of a host is usually divided into three steps: host-habitat location, host location, and host acceptance (Doutt, 1964). The processes of host-habitat location and host location are intensively studied in relation to optimal foraging (e.g., Waage, 1979) and biological pest control (e.g., Ridgway and Vinson, 1977). After locating the host habitat the parasite will have to decide how long it will stay in that particular host-habitat patch. This decision is assumed to be made among others on the basis of chemical and/or physical stimuli (e.g., Weseloh, 1981).

Our group has studied host-location processes in several parasites, most data

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being available for *Leptopilina heterotoma* (=*Pseudeucoila bochei*), a parasite of *Drosophila* species. Host-habitat location was discussed by Bakker and Van Lenteren (1979), Van Lenteren and Bakker (1978, 1979), and Dicke et al. (1984), while host acceptance was discussed by Van Lenteren (1976), Van Lenteren and Bakker (1979), and Carton et al. (1985). The combined data give us an idea of the entire process of host-habitat location, host location, and host acceptance of one species, whereas most knowledge about this process until now is confined to its separate aspects in different parasite species.

A parasite that has reached the host habitat can determine if hosts are present by physical or chemical stimuli produced by the host. In recent years more and more evidence has been gathered that chemical stimuli play an important role in host location. Most often, though, the so called kairomones (Brown et al., 1970; Nordlund and Lewis, 1976) have not been identified (e.g., Corbet, 1971; Waage, 1978; Giron, 1979). Some exceptions are Hendry et al. (1973), Mossadegh (1980), Jones et al. (1973), Vinson et al. (1975), Lewis et al. (1982), Mudd and Corbet (1982).

We know that females of *L. heterotoma* can recognize (previous) presence of hosts in a yeast spot: they stay longer on such a spot and probe more frequently (Van Alphen et al., 1984), but the stimulus on which this recognition is based could not be deduced from these experiments. Since *Asobara tabida,* another parasite of *Drosophila* larvae, recognizes a water-soluble kairomone produced by the hosts (Galis and Van Alphen, 1981), research was started to determine whether *L. heterotoma* also recognizes a kairomone produced by *Drosophila* larvae.

In this paper results are presented on the presence and role of kairomones in the intensification and prolongation of host searching in *L. heterotoma,* as well as on the purification of the kairomone.

## METHODS AND MATERIALS

*Hosts and Parasites.* The hosts were larvae of *Drosophila melanogaster* (strain WW), reared in our laboratory for 20 years. For rearing methods see Bakker (1961). In all experiments larvae were used that had emerged 24 hr before and which were maintained at 25°C.

The parasite, *L. heterotoma* (strain Storrs) was obtained from the United States and has been reared in our laboratory for 12 years. For rearing methods, see Bakker et al. (1967).

Experienced (Van Lenteren, 1976) 7- to 14-day-old parasites that had been maintained at 13°C were used in all experiments.

*Host Habitat.* Our experimental host habitat consisted of *Saccharomyces cerevisiae* (Hansen) ('Engedura' Gist Brocades, Delft, the Netherlands). A suspension (25 g living dry Engedura yeast per 100 ml water) was used. From this

suspension 0.50 ml was poured into a Perspex ring (inside diameter 2.1 cm) lying on agar (2 or 4%) in a Petri dish (diameter 5 cm). The superfluous water was evaporated by placing the Petri dish in the air stream of a ventilator. After removal of the Perspex ring, a "yeast spot" was obtained.

*Bioassay.* A bioassay was developed to study the reaction of *L. heterotoma* to a kairomone possibly produced by *D. melanogaster.* It appeared that yeast is necessary for any reactions to host-related stimuli by *L. heterotoma* to occur. The specific short-range host-searching behavior of *L. heterotoma* is a rhythmical pricking with the ovipositor in the substratum. For the bioassays, we prepared yeast spots of different quality: yeast spots possibly contaminated with a kairomone and control yeast spots. Both yeast spots were identical in diameter and quantity of yeast, and their surface was smooth.

When a parasite was placed in a Petri dish containing a yeast spot, it readily examined this spot, because the parasite is attracted to yeast (Dicke et al., 1984). If, in an experiment, a parasite left the yeast spot for 20 sec or more, the experiment was terminated. This value was determined during preliminary experiments: if the parasite leaves the yeast for such a period she usually does not return but walks to the lid of the Petri dish. Leaving for a shorter period often resulted in returning to the yeast spot. The total time of searching on the yeast spot and excursions shorter than 20 sec was taken as examination time. If the parasite did not start to examine the yeast spot within 10 min after introduction into the Petri dish, the experiment was terminated and the parasite was not used anymore. After a first visit to a yeast spot, the parasite was not used for at least 30 min. Then she was offered a second yeast spot of the other category. About 50 parasites were offered a control yeast spot and a test spot, respectively. Fifty other females were offered yeast spots in the reverse sequence. About 12 females were used per individual yeast spot. In the Results section this part of the procedure will be discussed in relation to the possibility of the parasites marking the examined yeast spot.

*Preparation of Yeast Spots of Different Quality.* A number of procedures was used to try to isolate and purify a possible kairomone.

1. A yeast spot in which 64 *Drosophila* larvae had been present during 20 hr at 25°C was extracted with 1.0 ml of water and filtered over filter paper. The filtrate was added to 0.50 ml of yeast suspension and poured in a Perspex ring on an agar base to prepare a new yeast spot as described above. The control yeast spot was prepared using the same procedure but starting with a yeast spot without hosts that had also been incubated at 25°C for 20 hr.

2. A chloroform extract of a yeast spot with 64 *Drosophila* larvae and a control chloroform extract were prepared using 1.0 ml of chloroform. The control and test extracts were added (dropwise) to a clean yeast spot after removing the Perspex ring.

3. The different components of the water and chloroform extracts (both test and control) were separated from each other using thin-layer chromatography

(TLC) on silica-gel coated plates (Merck 60F-254, thickness 0.25 mm). Water–ethanol (1:3 v/v) was used as solvent to analyze the water extract. Chloroform–petroleum ether (40–60)–acetone–acetic acid (20:20:5:1 v/v) was used in the case of the chloroform extract.

Different parts of the plates were extracted with water and filtered over filter paper. These extracts were used to prepare new yeast spots, both control and test spots. All yeast spots prepared as described above were used in bioassays.

*Effect of Host Number on Examination Time.* Yeast spots were prepared as described under Host Habitat. In these yeast spots varying numbers of hosts (1, 2, 4, 8, 16, 32, or 64) were introduced for a 20-hr period at 25°C. The yeast spots were extracted with water after this period and new yeast spots were prepared as described under Preparation of Yeast Spots of Different Quality. In this experiment only one parasite was tested per yeast spot. Fourteen to 53 females were tested at each host density.

## RESULTS

*Isolation of Kairomone.* For isolation of a kairomone, yeast spots with and without host larvae were extracted with water and chloroform. In the bioassay these extracts were examined. Table 1 shows that in both cases a first visit to a control yeast spot is shorter than a first visit to a test spot. For the second visits the results are identical. The second visit is shorter than the first visit in the sequence test–control but not in the sequence control–test spot. This means that the parasites do distinguish between test and control yeast spots. In other words a kairomone can be extracted using water or chloroform as solvent.

TABLE 1. MEAN EXAMINATION TIMES (SEC) OF *L. heterotoma* ON YEAST SPOTS OF DIFFERENT QUALITY

| | First visit | | | | Second visit | | | | Mann-Whitney U test ($P$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control spot time | $N$ | Test spot time | $N$ | Control spot time | $N$ | Test spot time | $N$ | First visit | Second visit |
| Water extract of a yeast spot | 188 | 53 | 320 | 50 | 71 | 47 | 241 | 46 | <0.001 | <0.001 |
| Chloroform extract of a yeast spot | 133 | 58 | 189 | 58 | 103 | 47 | 146 | 49 | <0.001 | <0.01 |

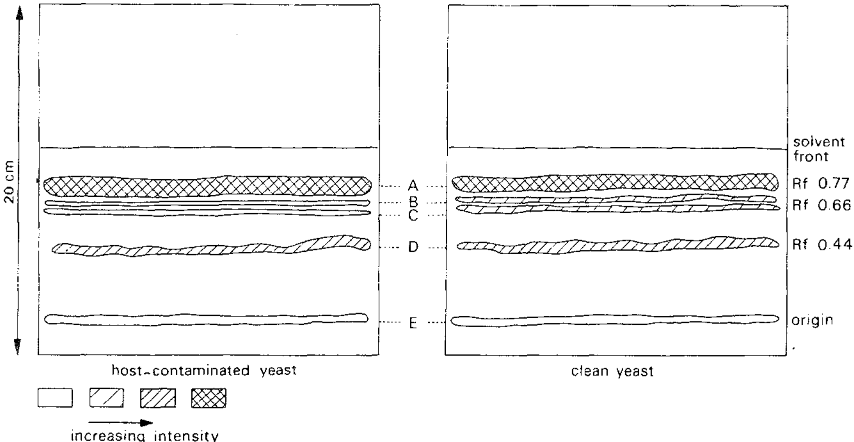


FIG. 1. Silica gel after semipreparative thin-layer chromatography of a complete water extract of yeast with hosts and of clean yeast.

*Separation of Components of Extracts.* The different compounds of the extracts were separated using TLC. The water extract was separated into five components that were visible under UV light, the chloroform extract in three components. No qualitative differences can be observed between extracts with and without host larvae, only quantitative differences (Figures 1 and 2).

*Effect of Different Components of Extracts on Searching Time.* Several of the components obtained through TLC were tested in the bioassay for their capacity to increase searching time. The results of these tests are presented in Table 2.

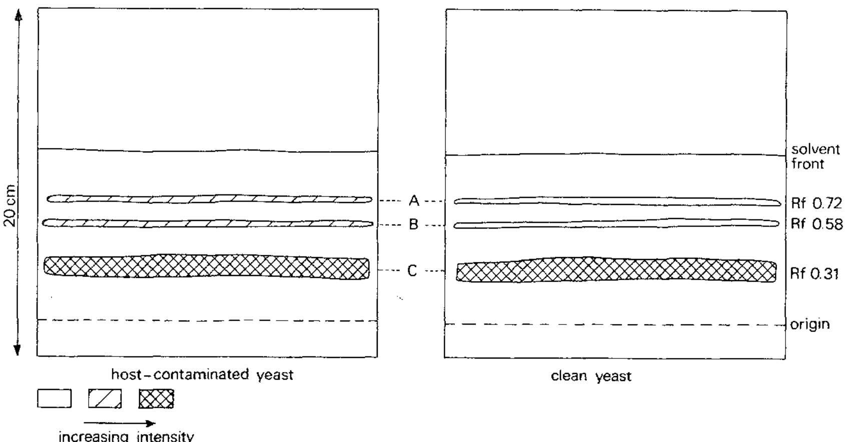


FIG. 2. Silica gel after semipreparative thin-layer chromatography of a complete chloroform extract of yeast with hosts and of clean yeast.

TABLE 2. MEAN EXAMINATION TIMES (SEC) OF *L. heterotoma* ON YEAST SPOTS TO WHICH WATER EXTRACT OF SILICA GEL IS ADDED

| Water extract of silicagel | First visit | | | | Second visit | | | | Mann-Whitney U test ($P$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control spot time | $N$ | Test spot time | $N$ | Control spot time | $N$ | Test spot time | $N$ | First visit | Second visit |
| $R_f$ 0.66 (sample TLC: water extract of yeast spot) | 244 | 31 | 271 | 30 | 153 | 28 | 169 | 22 | N.S. | N.S. |
| $R_f$ 0.00 (sample TLC: water extract of yeast spot) | 147 | 53 | 177 | 51 | 114 | 47 | 183 | 45 | $<0.05$ | $<0.001$ |
| $R_f$ 0.58 + $R_f$ 0.72 (sample TLC: chloroform extract of yeast spot) | 105 | 59 | 107 | 52 | 81 | 24 | 87 | 28 | N.S. | N.S. |
| $R_f$ 0.00 (sample TLC: chloroform extract of yeast spot) | 116 | 59 | 132 | 60 | 116 | 34 | 107 | 40 | N.S. | N.S. |

With the water extract, when using the components that have $R_f$ values around 0.66 (B and C together, Figure 1), no differences in searching time could be observed between control and test spots. Also the second visit was shorter than the first visit of the parasites. In the case of component E ($R_f$ 0.00), the parasites distinguished a test spot from a control spot. In this case the second visit is not shorter than the first in the sequence control–test spot. With the chloroform extract, the components A and B were tested in the bioassay ($R_f$0.58 + $R_f$ 0.72, Figure 2), but the parasites did not distinguish test spots from control spots when these compounds were applied, nor when the compounds present on the origin ($R_f$ 0.00) were tested. Also in these tests the second visits were shorter than the first for both sequences of offering the yeast spots of different categories.

*Effect of Visits of Conspecifics on Searching Time.* In the bioassay several females were tested per yeast spot. This was done because the procedure of preparing the yeast spots is very laborious. The following preparations were made prior to a bioassay: a yeast spot with or without hosts was incubated; this incubated yeast spot was extracted; the extract was developed with TLC and finally one component of the TLC plate was extracted to prepare a new yeast spot with this extract. To see whether the previous visit of conspecifics influences the searching time, a graph is made in which the time spent by an individual parasite is plotted against the total time spent by conspecifics during previous visits (one example is given in Figure 3), and the correlation coefficients for all different situations were calculated (Table 3). If parasites apply a mark to the yeast spot (in order to be able to limit the time spent searching such sites at later visits), this would mean that the visits should become shorter with increasing total visit time. Of all 12 experiments, only three have a correlation coefficient that significantly differs from zero, one correlation coefficient is positive and two

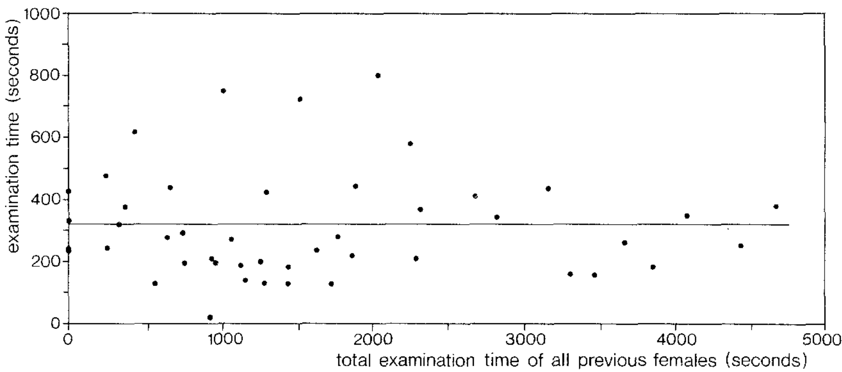


FIG. 3. Examination time (seconds) of individual *L. heterotoma* females of a first visit to a yeast spot (in this example a water extract of a yeast spot with hosts is present) plotted against the total examination time of all previous visiting females on the same yeast spot. Line: calculated linear regression line.

TABLE 3. INFLUENCE OF PREVIOUS VISITS OF CONSPECIFICS TO SAME YEAST SPOT ON EXAMINATION TIME OF A *L. heterotoma* FEMALE[a]

| Extract tested | Yeast spot | Correlation coefficient | N |
|---|---|---|---|
| Water extract of a yeast spot | control | 0.01 | 53 |
| | test | 0.03 | 50 |
| Water extract of silica gel, $R_f$0.00 (sample of TLC: water extract of yeast spot) | control | 0.38[b] | 53 |
| | test | 0.08 | 51 |
| Water extract of silica gel, $R_f$0.66 (sample of TLC: water extract of yeast spot) | control | 0.01 | 31 |
| | test | −0.41[b] | 30 |
| Chloroform extract of yeast spot | control | −0.36[b] | 58 |
| | test | 0.11 | 58 |
| Water extract of silica gel $R_f$ 0.00 (sample of TLC: chloroform extract of yeast spot) | control | 0.10 | 59 |
| | test | 0.05 | 60 |
| Water extract of silica gel $R_f$ 0.58 + $R_f$ 0.72 (sample of TLC: chloroform extract of yeast spot) | control | 0.13 | 59 |
| | test | 0.01 | 52 |

[a]Presented is the correlation between examination time of a *L. heterotoma* female visiting a yeast spot for the first time and the total examination time of all previous visits to that same yeast spot.
[b]Correlation coefficient differs significantly from zero ($P < 0.05$).

are negative. Apparently previous visits do not influence the visit time of other parasites.

*Effect of Host Number on Kairomone Concentration.* Yeast spots in which different numbers of hosts had lived were extracted with water and new yeast spots were made using the extracts. In Figure 4 the average searching time (with standard deviation) is plotted against host number. The data for the clean yeast spot and the one with 64 hosts were obtained from the experiment on isolation of the kairomone (Table 1, water extracts).

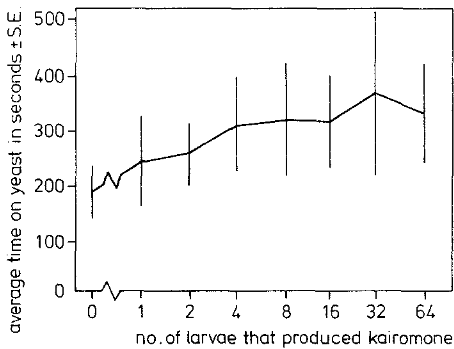


FIG. 4. Examination time on yeast spot (in seconds, with standard deviation) against the number of larvae that produced the kairomone during a 20-hr period.

Parasites search significantly longer on host-contaminated yeast spots than on clean ones (Mann-Whitney U test $P < 0.05$), and the increase in searching time with the increase of the number of hosts that had stayed in a yeast spot is also significant (Terpstra's nonparametric test for trends in a number of aselect samples, $P < 0.00003$; De Jonge, 1958). Without actually contacting hosts, the parasites are able to obtain information on presence or absence of hosts, as well as on quantitative aspects: the amount of kairomone. A certain amount of kairomone may be produced by combinations of different numbers of hosts present during different periods.

## DISCUSSION

Previous experiments have shown that *L. heterotoma* can determine whether host larvae have been present in a yeast spot (Van Alphen et al., 1984), but in those experiments host larvae were removed shortly before introduction of the wasp. In such a yeast spot the wasps could, besides picking up information from kairomones, also touch cast larval skins or insoluble parts of feces and perceive crawling tracks. In our experiments only soluble chemicals may have caused changes in searching time, and this shows that a kairomone plays a role in recognition of host presence. This kairomone is soluble in water and chloroform. After TLC of a water extract of a host-containing yeast spot, the (or an) active compound is present on the origin. The active compound present in the chloroform extract is not found after TLC in the parts of the plate that were examined.

The fact that the kairomone can still be recorded after TLC of the water extract indicates that the compound is a stable molecule as it has undergone many treatments including freezing, thawing, being at room temperature for a long time (half a day to a day), etc. It is known that parasites of some species mark an area in which they search for hosts so that it can be recognized afterwards (e.g., Galis and Van Alphen, 1981; Price, 1970). It seems that *L. heterotoma* females do not apply a mark which can be recognized by individuals of the same species since, when several parasites are tested on the same yeast spot, there is no reduction in the examination time. In experiments where the same female visited the yeast spot several times, a decreasing examination time was measured (Van Lenteren and Bakker, 1978), this decrease is either effected by an intraindividual marking substance or by a decrease in searching tendency of such females.

Very few kairomone structures are elucidated to date. Hendry et al. (1973) report that heptanoic acid is a kairomone that is recognized by *Orgilus lepidus*. Tricosane (Jones et al., 1973), 13-methylhentriacontane (Jones et al., 1971), and combinations of monomethylhentriacontanes, monomethyldotriacontanes, and monomethyltritriacontanes (Vinson et al., 1975) are kairomones that belong to the alkanes. Henson et al. (1977) report that a cholesterol ester is an oviposition stimulus for *Bracon mellitor*.

Very little is known about chemical communication in *Drosophila.* Recently Jallon et al. (1980) and Antony and Jallon (1982) studied the chemical basis of sex recognition in *D. melanogaster.* These studies demonstrated that heptacosadiene, a long-chain hydrocarbon, is the main aphrodisiac pheromone. This compound is one of the cuticular hydrocarbons of *D. melanogaster* that comprise *n*-alkanes, alkenes, alkadienes, and 2-methylalkanes [with 21, 23, 25, 27, or 29 carbon atoms (Jackson et al., 1981; Antony and Jallon, 1982)]. The cuticle of *Drosophila* larvae, however, contains small amounts of hydrocarbons, perhaps because the larvae do not need protection against drought (L. L. Jackson, personal communication).

Nothing is known about the structure of the *Drosophila* kairomone so far. A GC-MS analysis of the purified kairomone was unsuccessful. Not much can be said regarding the site of production of the kairomone. Kairomones are reported to originate from different places, like cuticle (Jones et al., 1971) or mandibular glands (Waage, 1978). In these cases the amount of kairomone will be much smaller than when it is a component of the feces (Henson et al., 1977). The same reasoning holds for the pheromones that can act as a kairomone (e.g., Mossadegh, 1980). Pheromones are usually being produced in nanogram or picogram quantities (Tumlinson and Heath, 1976). To elucidate the production site of the kairomone that is produced by *D. melanogaster* further research is necessary.

We have shown that *L. heterotoma* can distinguish a host-contaminated yeast spot from a clean yeast spot and that the presence of a kairomone increases the visit time to a patch. Furthermore, the visit time increases when the amount of kairomone increases. We have previously found that visit times are influenced by the size of the yeast spot and by the number and quality of hosts present (Bakker and Van Lenteren, 1979; Van Lenteren, 1976; Van Lenteren and Bakker, 1976, 1978). The information presented in this paper shows that this parasite is also able to obtain information about the quality of a patch without contacting hosts. The same ability was found recently in another parasite of *Drosophila, A. tabida* (Galis and Van Alphen, 1981). Parasites may use it as one of the mechanisms to allocate patch time (see Waage, 1979). In this context it will be interesting to find out whether the kairomone is host-species specific, because then the parasites might even be able to select between yeast spots contaminated by different host species.

Another interesting question is whether the kairomone to which *A. tabida* reacts is the same (mixture of) compound(s) as the one to which *L. heterotoma* reacts. In both parasites a host-recognition system has evolved independently. If they both react to the same kairomone, this would indicate that the kairomone is a specific compound for *Drosophila* and not for other arthropods living in the same habitat. Structure analysis of the kairomone will shed light on this matter.

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# INTERSPECIFIC ATTRACTIVITY OF FEMALE SEX PHEROMONE COMPONENTS OF *Periplaneta americana*

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**Abstract**—Female sex pheromones of *Periplaneta americana, P. australasiae,* and *Blatta orientalis* elicit locomotion and upwind orientation in conspecific males. Interspecific attraction between these species was observed in a wind tunnel assay for most species combinations, with the exception of *P. australasiae* males not being attracted to *P. americana* females. The role of two *P. americana* sex pheromone components, periplanone-A (PA) and periplanone-B (PB) in male attraction was analyzed. PA induced locomotion in males of all three species, with highest threshold concentration in *P. americana*. PB was effective in *P. americana* and *B. orientalis,* but not in *P. australasiae.* Experiments with mixtures of both components confirmed an inhibitory influence of PB on *P. australasiae* males when it was added to PA in a ratio of 3 : 1 or higher. Since *P. americana* and *P. australasiae* have been found to be syntopous, and their daily mating periods overlap, the female sex pheromone of *P. americana* is discussed as an isolating factor between both species.

**Key Words**—Cockroach, *Periplaneta*, Orthoptera, Blattidae, sex pheromone components, interspecific attraction, isolation mechanisms.

## INTRODUCTION

The closely related cockroach genera *Periplaneta* and *Blatta* originally lived in the Old World tropics where some of the numerous species were sympatric while others were widely separated (Cornwell, 1968). Several species have been introduced by man to most tropical and subtropical countries in the world where they overlap in distribution and habitat (Roth and Willis, 1960). Cross-breeding seems to be very exceptional, hence species-isolating mechanisms must be postulated.

Simon and Barth (1977a) studied the mating behavior of five *Periplaneta* and one *Blatta* species. All six species have similar courtship behavior and use



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female sex pheromone for distance attraction and male tergal gland secretions during the precopulatory courtship sequence. Simon and Barth (1977b) investigated male responses to female odors and found interspecific effects in all species combinations they tested. They concluded that the female sex pheromones of all species must be similar and cannot provide the cues for species recognition. The authors ascribe this function to the male tergal gland secretions enabling female choice of conspecific males and repulsion of heterospecific ones by aggressive acts.

Recent studies indicate that the female sex pheromone system of *Periplaneta* is more sophisticated than assumed by earlier authors. Sass (1983) found two distinct male olfactory receptor types specialized for the perception of two female-produced pheromone components, periplanone-A (PA) and periplanone-B (PB) in *Periplaneta americana.* Both components are released by the females in approximately equal amounts (Sass and Seelinger, in preparation). PB but not PA caused distance attraction of *P. americana* males in a tropical outdoor environment, while males of the syntopous species *P. australasiae* were attracted to PA and repelled by PB (Waldow and Sass, 1984). Virgin *P. americana* females attracted only conspecific males at the same experimental sites (Seelinger, 1984). It appears that quantitative studies of female odor composition and male responsiveness to pheromone components as well as field studies with live females and artificial pheromone sources are necessary in order to understand the function of the *Periplaneta* sex attractant system.

The present paper provides a quantitative study of interspecific pheromone action in cockroaches. Three species were selected: *P. americana*, because the purified main components of the female odor were available; *P. australasiae*, because it probably originated from the same Old World region as *P. americana* (Cornwell, 1968) and field data on a mixed population of both species indicate a highly specific attraction system; and *Blatta orientalis*, because it may provide an example of an originally allopatric species which only recently was confronted with *P. americana* in certain anthropogenic environments (Roth and Willis, 1960). Responses of males to females of all three species and to the *P. americana* sex pheromone components are described and discussed with respect to their possible role as isolation factors. In addition, the daily mating cycle of each species was investigated to make sure that the problem of interspecific attraction is not circumvented by temporal segregation of these species.

## METHODS AND MATERIALS

The *P. australasiae* culture was based on specimens captured in Jamaica in 1982, while the cultures of both other species are of unknown geographical origin and have been kept in the laboratory for many years. Male and female cockroaches used for tests were selected from stock on the day of their imaginal

ecdysis. They were kept at 27 ± 2°C under a 12:12 light–dark photocycle, fed with commercial dry dog food and supplied with water ad lib. Males were kept separated from females and from female odors. Experiments were performed during the daily mating period of the respective species (see results).

*Pheromones and Stimulus Quantification.* Two pheromone components of the female odor, periplanone-A (PA) and periplanone-B (PB), were kindly provided by Dr. H. Sass. They were extracted from filter papers sampled in cages of virgin females with hexane and purified by HPLC chromatography. Details of the isolation and purification process can be found in Sass (1983). PB was identified by chromatographic comparison with synthetic material (provided by Dr. W.C. Still) and its effectiveness in the electroantennogram (EAG) and behavioral tests. PA corresponds to the most active component in the chromatographic "A region" of Persoons (1977). A proposed chemical structure has not yet been confirmed by successful synthesis. Quantification of pheromone amounts by direct measurements was not possible, due to the low quantities available. Scaling of stimulus strength was therefore achieved by reference to its effectiveness in sensory stimulation of the male antenna. Dilution series of PA and PB were compared to known amounts of synthetic PB in the EAG (Sass, 1983). Quantities eliciting the same EAG amplitude as 1 ng synthetic PB racemate are referred to as 1 ngEq (nanogram-equivalent) of natural PA or PB.

For stimulus application in the behavioral assays, 50 $\mu$l of pheromone solution were dispensed on a 2-$cm^2$ filter paper and the solvent (hexane) was allowed to evaporate. The filter paper was then introduced into the test situation as described below. The evaporation rate of pheromone from the filter paper is not known exactly. Data concerning stimulus strength therefore refer to the amount of pheromone applied to the filter paper.

*Determination of Daily Mating Period.* Pairs of one virgin female and one conspecific male were placed in 11 plastic containers on the day of the female's imaginal ecdysis and kept under a 12:12 light–dark photocycle. Groups of 10–16 pairs were continuously observed by video with a time-lapse recording (1 frame/5 sec) until 90% or more of the females were mated. Mating was easily detectable by the opposed position of the pair which lasted for 1–2 hr. The beginning of each copulation was noted.

*Attraction Tests with Virgin Females in a Small Wind-Tunnel Apparatus.* Virgin females (2–4 weeks old) were accustomed to 10 × 10 × 5 cm wire grid cages with a cardboard bottom for several days. During a test they were placed at the upwind end of a wind tunnel of 130 × 50 × 50 cm (described by Seelinger and Schuderer, 1985) which provided constant and even airflow of 15 cm/sec. A single male was confined in a wire grid cage and accustomed for 10 min. Then the cage was placed in the wind tunnel 80 cm downwind from the female and opened on its upwind side. Upwind runs along the midline of the wind tunnel (for 40 cm or more) and contacts of the male with the female's cage were recorded. The test was terminated after 3 min.

In a test series, a female was successively tested with five males of each species. The results were discarded when less than three conspecific males reached the female's cage, in order to avoid experiments with unreceptive females. Five test series were performed for each species. Red light was used for observation.

*Bioassay for Quantification of Locomotory Activity.* Groups of five male roaches were kept in 10-liter plastic containers and supplied with water, food, and perforated cardboard shelter. The wooden lids of the containers had six peripheral and one central opening of 32 mm diameter. Air was blown vertically down into the containers at a rate of 2 liters/min through 4-mm plastic tubing connected to the central opening. A pheromone-loaded filter paper was introduced into the airstream for a test. This assay is similar to those used by Wharton et al. (1954) and Block and Bell (1974), but the modified method of odor application provides shorter rise time of pheromone concentration and better clearance between successive tests than does suspension of a stimulus paper without airflow.

Stimuli were applied for 1 min. The method of Block and Bell (1974) was used to measure running activity. A black line was drawn across the middle of the container's bottom and the number of line-crossings by all roaches was counted during the time of stimulation. This value is called MAC (male activity counts) following Bell (1982). *P. americana* males were tested with several concentrations of one pheromone component in an ascending order at 20-min intervals. The males respond with a steep rise in the MAC value above threshold concentration. Control tests showed that responses to suprathreshold concentrations were not measurably influenced by previous stimulation with lower concentrations. In *P. australasiae* and in *B. orientalis*, in contrast, behavioral adaptation seriously affected the response to subsequent stimuli. Therefore, quantitative data were obtained by presentation of one stimulus on a day. Two-day intervals were observed between experiments with the same individuals.

*Flight in a Large Windtunnel.* Behavioral responses of *P. australasiae* males to mixtures of pheromone components were investigated in a wind tunnel of 5 × 2 × 2 m. The walls of the wind tunnel were made of white plastic material, the bottom was covered with black cardboard. The top consisted of window glass panels. A large fan created an airstream of 30 cm/sec which was evenly distributed over the whole cross-section by an iron-mesh and smoothed by a sheet of air-filter material. The fan was combined with a heater and a sensor adjusting the temperature to 28 ± 1°C.

The odor source was a pheromone-loaded filter paper on a tripod of 1 m height. It was positioned in the middle of the upwind end of the wind tunnel, 10 cm sideways of a tree trunk 20 cm thick and 1.8 m high which served as visual target and landing site for the flying males. The males were released on a platform 3 m downwind from the pheromone source. The platform connected to a cross-shaped board extending 1 m in the horizontal and 0.5 m in the vertical

plane. Males running along this cross could check the borders of the odor plume before they took flight.

The odor plume was initiated by application of $TiCl_4$ to the stimulus filter paper. It passed down along the midline of the wind tunnel and reached a width of about 50 cm at the release platform. The air flowing down the wind tunnel was circulating in a room of 10-$m^2$ total cross-section area and entered again at the upwind end. Considerable contamination should be expected without filtering or chemical destruction of the pheromone. However, males placed on the release platform never did show sexual excitation or increased locomotion prior to stimulus introduction, even when the previous stimulus had been 1000 times threshold concentration. Deposition on the air filter or chemical destruction in the heating section may be the reasons. In addition, the room was frequently ventilated between successive experiments.

The ceiling of the room was dimly illuminated by indirect light from normal 60-W white light bulbs, resulting in diffuse light of 1–3 lux in the middle of the wind tunnel. Flights were recorded with an infrared-sensitive TV camera from above and could be replayed in single frames for exact survey of the events. Diffuse infrared illumination was provided by a 300–W wide-angle IR source (Philips DVF 102) directed towards the ceiling.

For a test, five males were confined in a wire grid cage and placed on the release platform. The cage was opened after stimulus introduction, and the males were observed for 6 min. The number of males running on the platform, flying, or staying in the open cage was recorded, as well as the landing sites of the flyers. Males were used either only once in their lives, or twice with at least a 3-day interval. Their age was 2–4 weeks after imaginal ecdysis when tested.

## RESULTS

*Temporal Distribution of Mating Activity.* Each female mated only once during the observation time of ca. 2 weeks. The peak of mating activity was on days 4–6 in *B. orientalis*, days 6–8 in *P. americana*, and days 8–12 in *P. australasiae* females. A clear daily maximum in overall mating activity was apparent in all species. Matings of *P. americana* were observed in the second half of the light phase and in the first half of the scotophase, with a pronounced peak in hours 3 and 4 after dark (Figure 1A). Mating of *P. australasiae* and of *B. orientalis* was restricted to the night, with a peak around midnight. Overlap of the mating periods is seen in all species combinations (Figure 1). Hence, temporal segregation is not an effective mechanism for species isolation in this case.

*Attraction Experiments with Virgin Females in a Small Wind Tunnel. P. americana* males were strongly attracted when conspecific females were presented (Table 1). Females of other species were obviously more difficult to keep in a receptive condition since most of them started to produce oothecae in their

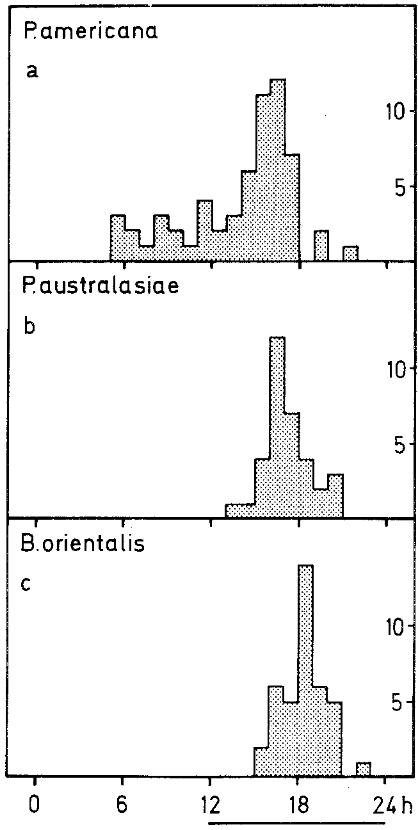


FIG. 1. Daily mating periods of three cockroach species. Ordinate: number of matings starting during the respective hours. Abscissa: time of the day; / the black bar indicates the scotophase.

TABLE 1. ATTRACTION OF MALES TO CON- AND HETEROSPECIFIC FEMALES FROM 80-CM DISTANCE IN A WIND TUNNEL[a]

| | Percent attracted | | |
|---|---|---|---|
| | *P.am.* ♂♂ | *P.aus.* ♂♂ | *B.or.* ♂♂ |
| *P. americana* ♀♀ | 100 | 0 | 68 |
| *P. australasiae* ♀♀ | 48 | 64 | 20 |
| *B. orientalis* ♀♀ | 32 | 60 | 68 |

[a]Each species combination was tested with five females and 25 males. Percentage of males having contact to the females' cage within 3 min are given. In a control series with 25 *P. americana* males and clean, empty cages, no contacts were registrated.

wire grid cages and did not attract conspecific males in the wind tunnel. Nevertheless, species-specific differences in attractiveness of those females were observed which did attract conspecific males. *P. americana* females attracted 100% of the conspecific males, but *P. australasiae* males never responded to them. In all other species combinations intermediate degrees of attraction were observed (Table 1). Evaluation of chemoanemotactic runs and of contacts with the female's cage yielded identical results.

*Experiments with Purified Pheromone Components (MAC Method).* The behavioral responses of *P. americana* males to sex pheromone components in this test are described in detail by Seelinger (1985). Both components elicit fast locomotion, increasing with stimulus concentration above threshold (Figure 2). Thirty times more PA was needed to equalize the effect of a certain PB concentration in behavioral tests than in sensory stimulation (EAG or single-cell response) (Sass, 1983).

The effectiveness of both components was different in the other species studied than in *P. americana.* Males of *P. australasiae* were very sensitive to PA. Their behavioral threshold for this substance was considerably below that of *P. americana*. Their locomotory activity, however, increased at a somewhat lower rate, which is at least partly due to their smaller size and their tendency to climb the walls of the containers and fly. PB had no detectable effect on *P. australasiae* males even in high concentrations (Figure 2). *B. orientalis* males responded to both components, PA being slightly more effective (Figure 2). Locomotory activity increased even more slowly than in *P. australasiae.* Qualitative differences in the response to PA and to PB were not observed.

*Attractivity of Sex Pheromone Blends for* P. australasiae. Field observations on Jamaica had shown that both *P. americana* and *P. australasiae* males usually approached a virgin female or a pheromone source in flight (Waldow and Sass, 1984; Seelinger, 1984). Waldow and Sass postulated an inhibitory influence of PB on *P. australasiae* males on the basis of attraction experiments with pure PA and a blend containing PB and PA in a ratio of 8 : 1 (in ngEq). Similar conclusions are suggested by the fact that *P. americana* females do not attract *P. australasiae* males in the field (Seelinger, 1984) or in the wind tunnel (see above), although they doubtlessly release the highly effective PA. The following experiments were designed to test this hypothesis under controlled experimental conditions for different ratios of both components. Since a possible inhibitory influence might affect a special part of the orientation process, a rather complex situation was used, including flight towards the pheromone source. Parameters for flight orientation will be described in a separate paper. Here, only some general remarks on the orientation behavior and a description of specific blend effects will be given.

Control experiments showed that no males of *P. australasiae* flew off the release platform without stimulation by PA. Most of them stayed within the open cage during the 5-min experimental time, while a few moved around slowly on

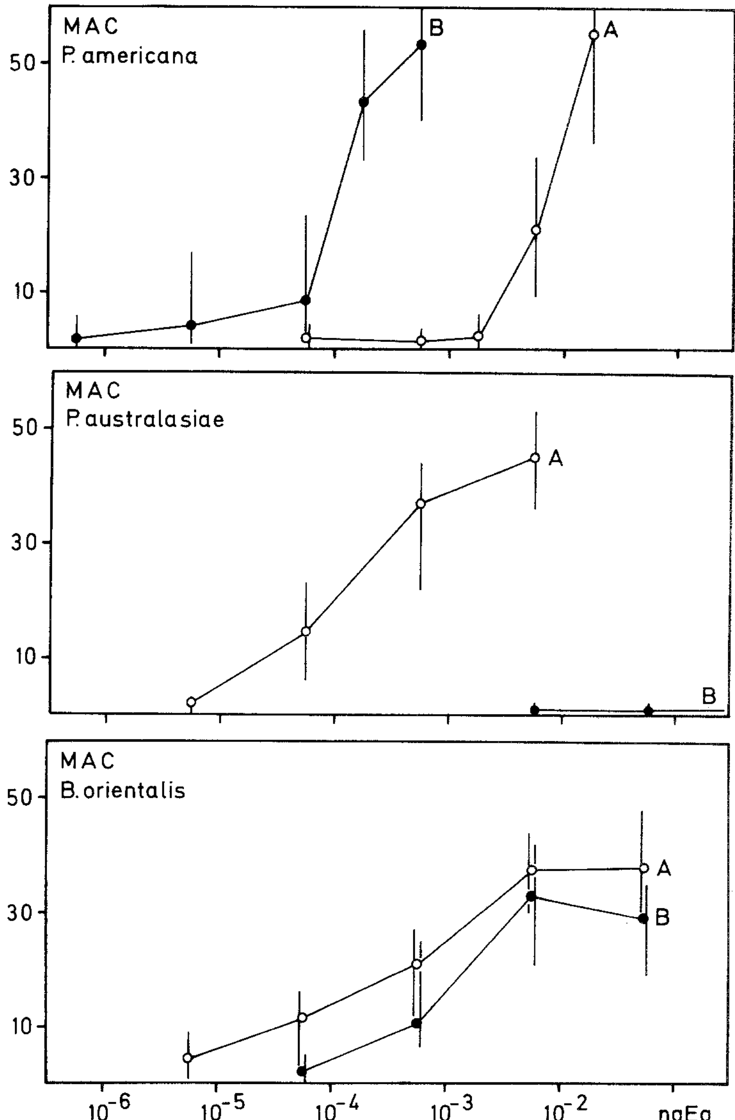


FIG. 2. Locomotory response (male activity counts) of three cockroach species to periplanone-A and periplanone-B. Ordinate: number of line-crossings by five males counted during 1 min of continuous stimulation. Abscissa: amount of pheromone applied on filter paper; for explanation of the scale see Methods and Materials. Median values of 30 (*P. australasiae, B. orientalis*) or 80 (*P. americana*) tests with different male groups are given. Vertical bars indicate 95% confidence intervals. All dose–response curves start from a level which is not different from control (hexane-treated filter paper).

the platform. When $1.5 \times 10^{-2}$ ngEq or more PA was applied to the stimulus filter paper, most males left the cage within a few seconds and either took off immediately or started running along the cross-boards before flying or calming down. Some individuals did not respond even to high pheromone amounts (Figure 3, upper row). A few males reached the floor by climbing down the release tripod.

The majority of flying males reached the tree trunk next to the pheromone

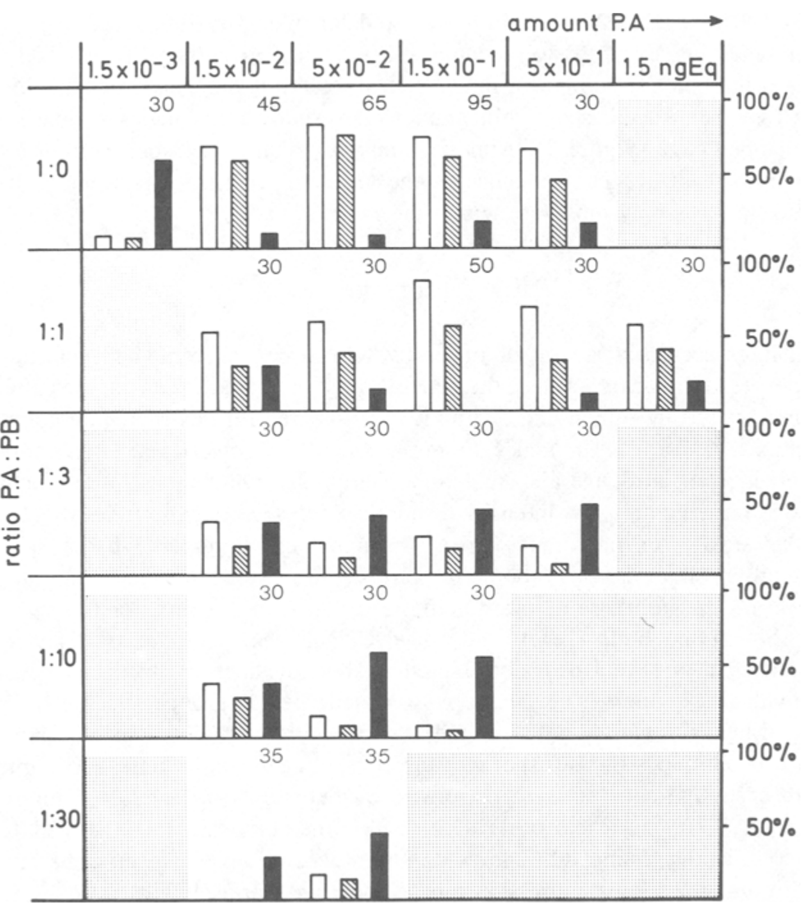


FIG. 3. Responses of *P. australasiae* males to periplanone-A alone and in mixtures with periplanone-B in a large wind tunnel with airborne pheromone plume. Lengths of the columns indicate the % of males alighting for flight (white column, left), landing on the target tree trunk (hatched column, center), or not responding (black column, right). The number of males tested is indicated in the upper right of each field. Shaded areas at far left and right indicate stimulus combinations not tested.

source. This was achieved either by direct, straight flight, after short inflight zigzagging in front of the pheromone source, or after repeated circling in the wind tunnel with many losses of the pheromone plume and eventual landings at the walls or ceiling of the tunnel. After landing on the tree trunk, they investigated it by running up and down, quite often starting a new flight downwind. Only 5% of the fliers landed on the pheromone source in the first attempt. Some terminated search after loosing the pheromone plume.

Few males responded to $1.5 \times 10^{-3}$ ngEq PA. Therefore, blends containing $1.5 \times 10^{-2}$ ngEq PA or more were tested for inhibitory influences of PB. No significant effects of PB were found at a ratio of 1:1. When PB was added at 3:1 or higher, the number of flying males and the number of males arriving at the tree trunk decreased significantly while usually more males stayed within their open cage (Figure 3). Almost complete inhibition was observed at a PB/PA ratio of 30:1. The influence of the absolute amount of pheromone blend presented was only moderate within the range tested.

## DISCUSSION

Interspecific attraction of males by female sex pheromones seems to be widespread in cockroaches of the genera *Periplaneta* and *Blatta*, as was already demonstrated by Simon and Barth (1977b). There are, however, important exceptions. *P. australasiae* males are not attracted to *P. americana* females due to interaction of attractive and inhibitory pheromone components. While Schafer (1977) reported only low-intensity responses of *P. australasiae* males to *P. americana* female sex pheromone extracts, Simon and Barth (1977b) observed a strongly positive response. Some reasons for this discrepancy between both authors' results and those reported here are included in this discussion.

Simon and Barth (1977b) did not investigate the specificity of distance attraction but of sexual behavior elicited at short distance or at contact between individuals. In their experiments with mixed species groups, release of male sexual behavior cannot be attributed to conspecific or to heterospecific females since pheromones of both species were present at the same time. Experiments with crude extracts, as were performed by Schafer (1977) and by Simon and Barth (1977b), may yield misleading results: the composition of the blend may be considerably changed by the extraction procedure with respect to the biologically important female emission rate. Simon and Barth (1977b) were aware of these facts. They modified their estimation of female sex pheromones as poor species isolation factors by admitting that "small differences in the female sex pheromones . . . may in conjunction with other behavioral and ecological factors function somewhat more effectively in nature."

Now we may consider the relative importance of possible isolation factors between the three species studied. (A) The daily mating cycle does not provide

a means of complete temporal segregation, but may contribute to species isolation, as was reported for some moth species (e.g., Grant, 1977; Collins and Tuskes, 1979). (B) Spatial segregation in syntopous populations of *P. americana* and *P. australasiae* is incomplete (Seelinger, 1985); appropriate field data for *B. orientalis* are not available. (C) Female volatile sex pheromone was shown to be an effective isolation factor in at least one species combination. It may not be so in others, especially when species were originally allopatric. Final evidence can only arise from field experiments with virgin females, since the effect of a pheromone blend depends on the exact relation of its components and an adequate environment. (D) Male sex pheromones were estimated highest in effectiveness as isolating factors by Simon and Barth (1977b) because they specifically inhibit aggression in conspecific females. However, in an outdoor aggregation of several thousand *P. americana* and *P. fuliginosa*, no interspecific courtships were seen among numerous intraspecific ones (Simon and Barth 1977b). This indicates a species recognition mechanism on the side of the males. (E) Other possible mechanisms discussed by Simon and Barth (1977b) are copulation time, genital organ compatibility, and genetic compatibility. These, however, should not normally play a role when two insect species are well-adapted to coexistence in the same habitat.

The pheromone system of *Periplaneta* and *Blatta* shows great similarities to multicomponent systems of moths (Inscoe, 1977; Kramer, 1978; Roelofs, 1979). The components are perceived by distinct, specialized receptor types on the male antennae (*P. americana*: Sass, 1983; *P. australasiae:* Beckmann, personal communication; moths: e.g., Kaissling, 1979). They can be attractive or inhibitory to heterospecific males (moths: e.g., Tumlinson and Teal, 1982). Further studies must show whether multicomponent pheromone systems of cockroaches function in the same way in intraspecific communication as they do in some moth species (Cardé et al., 1975; Bradshaw, 1983).

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# COMPARISON OF PONDEROSA PINES AS FEED AND NONFEED TREES FOR ABERT SQUIRRELS

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**Abstract**—Twigs from five ponderosa pine trees (*Pinus ponderosa*) used by Abert squirrels (*Sciurus aberti*) as feed trees and five nonfeed trees were collected every 45 days and their monoterpenoid and nutrient content determined. The *t* tests (unpaired observations) detected no significant difference in the level of monoterpenoids in the outer bark of feed (0.77%) and nonfeed (0.75%) trees. The same was true for inner bark of feed (0.10%) and nonfeed (0.16%) trees. Monoterpenoid levels in outer bark (0.75%) were significantly higher than inner bark (0.13%). The inner bark is what is eaten by Abert squirrels. Protein and other nutrients did not differ significantly between feed and nonfeed trees. However, both outer and inner bark were easier to remove from the woody portion of the feed tree twigs than those twigs collected from nonfeed trees. Therefore, due to the lack of differences in monoterpenoid and nutrient content between feed and nonfeed trees, we attributed the use of certain trees for use as feed trees to the ease of peeling and separating outer from inner bark.

**Key Words**—Monoterpenoids, crude protein, Abert squirrel, *Sciurus aberti*, ponderosa pine, *Pinus ponderosa*.

## INTRODUCTION

Abert squirrels (*Sciurus aberti*) have an obligate relationship with ponderosa pine (*Pinus ponderosa*) (Patton and Green, 1970; Pederson et al., 1976; Hall, 1981). Food items for the squirrel are produced directly or indirectly by ponderosa pine (Hall, 1981), including ponderosa pine seeds, inner bark (composed

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of phelloderm, phloem, cambium, and trace amounts of xylem), terminal buds, and fruiting bodies of fungi that grow in ponderosa pine duff and mistletoe berries. Major food items in the diet of the squirrel are fruiting bodies of fungi, terminal buds, and inner bark (Stephenson, 1975; Hall, 1981), that of the upper branches being preferred (Pederson et al., 1976; Farentinos et al., 1981; Hall, 1981). A feeding squirrel climbs out to the tip of a branch and cuts off a needle-bearing twig 8–10 cm back from the needles. Next, the squirrel chews off the needle cluster and allows it to fall. After removing the needle cluster, the outer bark is removed with the teeth while the twig is rotated with the forepaws. This exposes the phelloderm, which the squirrel eats along with the phloem, cambium, and trace amounts of xylem (Farentinos et al., 1981; Hall, 1981). Because Abert squirrels cache little, if any, food, they become totally dependent on terminal buds and inner bark during winters of heavy snow (Stephenson, 1975; Stephenson and Brown, 1980; Hall, 1981). Stephenson (1975) suggested that prolonged diet of terminal buds and inner bark may cause physical deterioration and mortality. Later Stephenson and Brown (1980) reported a strong correlation between annual mortality and number of days of snow cover of 10 cm or greater. Inner bark may contain insufficient nutrients to maintain the squirrels for extended periods.

Within a particular site, Abert squirrels select certain trees to feed on while totally excluding others[2] (Coughlin, 1938; Pederson et al., 1976; Hall, 1981; Farentinos et al., 1981). Farentinos et al. (1981) reported that captive Abert squirrels under laboratory conditions distinguished between twigs offered from feed trees and from known nonfeed trees. Differential preference among plants of the same species has been reported for mule deer (*Odocoileus hemionus*) (Sheehy and Winward, 1981; Welch et al., 1983), black-tailed deer (*Odocoileus h. columbianus*) (Radwan and Crouch, 1978), pygmy rabbit (*Brachylogus idahoensis*) (White et al., 1982), sage grouse (*Centrocerus urophasianus*) (Remington, 1983), and domestic sheep (Sheehy and Winward, 1981). Some workers have related differential preference to monoterpenoid content (Schwartz et al., 1980; Farentinos et al., 1981; Remington, 1983), others have not (Scholl et al., 1977; Radwan and Crouch, 1978; White et al., 1982; Welch et al., 1983). Therefore, we undertook this study to determine the role that monoterpenoids, nonstructural carbohydrates, and the components of proximal analysis play in Abert squirrel feed-tree selection. Also, the nutritive quality of inner bark was examined.

## METHODS AND MATERIALS

*Study area.* The study site—in the Devil's Canyon drainage of the Abajo Mountains—is 12 km southwest of Monticello, Utah, in San Juan County. Pon-

[2]These observations are for the consumption of inner bark. After seven years of field observations in the study area, we have noted no tree selectivity on the part of the squirrels for terminal buds or pine seeds.

derosa pine is the dominant tree species. Dominant understory species are snowberry (*Symphoricarpos* spp.), Gambel oak (*Quercus gambelii*), and quaking aspen (*Populus tremuloides*). A uniform 8-hectare site supporting a population of Abert squirrels was chosen to furnish feed and nonfeed trees. This area is a gentle, east-facing slope. The 20-year annual precipitation average (1952–1971) is 35 cm. The temperature extremes vary from −27°C to 36°C. Three soil series are found on the site: Monticello very fine sandy loam, depth 152 cm; Vega clay loam, depth 127 cm; and Abajo cobby loam, depth 127 cm.

On April 17, 1980, we selected and tagged five feed trees and five nonfeed trees within the study site. The trees were from the same age (110 years) and size class (40 cm mean diameter at breast height and a mean of 14 m of height). From these 10 trees, upper branches, the preferred feeding sites of Abert squirrels, were collected on April 17, May 25, July 6, August 17, October 9, and December 25, 1980; and January 25, March 15, and May 9, 1981. Branches were cut with a rope-pull tree pruner mounted on an 8-m extension pole.

Needle-bearing twigs were cut off 8–10 cm back from the needle cluster. Next the needle-cluster end of each twig was removed, leaving a bare twig 8–10 cm in length and about 3–5 years old. It took 40 of these twigs per tree per sampling date to yield enough tissue for the chemical analysis. After collecting, we placed the twigs in plastic bags that were labeled, tied, packed in crushed ice, and transported to a laboratory freezer (−35°C). Next, the twigs were thawed one set at a time and inner and outer bark removed and separated. As soon as the two bark components were removed and separated from a twig, we placed them into individual stainless-steel, wide-mouth, quart thermos bottles containing 500 ml of liquid nitrogen.

After all 40 twigs from a tree had been debarked, the bark samples were ground individually to a fine powder. Grinding was accomplished by placing the bark sample inside the mortar of a steel, motorized mortar and pestle. Liquid nitrogen was used to precool the mortar and then poured over the bark sample. After grinding, we placed the bark samples in plastic bottles fitted with airtight caps and stored them at −35°C.

We analyzed all bark samples quantitatively for: (1) total monoterpenoids, (2) individual monoterpenoids, (3) nonstructural carbohydrates (such as sugars, starch, and pectic acid), and (4) components of proximal analysis.

Monoterpenoids were extracted in Soxhlet extraction apparatus with absolute ethyl ether. For each sample, we placed 10 g of freshly ground tissue in a cellulose Soxhlet extraction thimble. A fiberglass plug on top of the sample inside the thimble prevented spillover of tissue during the extraction process. Monoterpenoids were exhaustively extracted from samples over a 6-hr period. Next, the volume of the extract was reduced to about 30 ml by use of reduced pressure. We added an internal standard, carvone (2.5 mg/$\mu$l), to each extract, then added absolute ethyl ether to bring the volume to 50 ml. Extracts were stored in airtight bottles at −35°C until needed for chromatographic analyses.

We made chromatographic analyses with a 5830A Hewlett-Packard flame

ionization, reporting gas chromatograph.[3] Monoterpenoids were separated by use of a $\frac{1}{8}$-in. × 4-ft stainless-steel column packed with 10% Carbowax 20 M on 80–100 Chromosorb WHP. We used a temperature program to separate individual monoterpenoids (Welch and McArthur, 1981) and identified monoterpenoids through retention times of standards. Dry matter content of each sample was determined, and the concentration of individual monoterpenoids was expressed as a percentage of dry matter. Total monoterpenoids are then a summation of individual monoterpenoids.

Nonstructural carbohydrate analyses were based on an $\alpha$-amylase enzyme digestion system followed by colorimetry using Teles' reagent (da Silveira et al., 1978). Amount of nonstructural carbohydrates is a measurement of available energy in a food.

After completing the monoterpenoid and nonstructural carbohydrate analyses, we performed proximal analyses of all bark samples. Proximal analysis, a method for judging the nutritive value of a food, is a series of chemical analyses that determines the crude protein, crude fat, crude fiber, and ash content of a food on a dry matter basis (Association of Official Analytical Chemists, 1980). A fifth class, called nitrogen-free extract, is determined nonchemically by subtracting the percentages of the other components from 100 (Welch 1983). Trees with significantly higher levels of crude protein, nitrogen-free extract, and lower levels of crude fiber are considered nutritionally superior.

During the process of peeling the twigs and separating the inner and outer bark for the first sampling date, we noticed a discernible difference in the difficulty of peeling the twigs between feed and nonfeed trees. To test the hypothesis that twigs from feed trees are easier to peel and to separate inner and outer bark than nonfeed trees, we conducted a test as follows: Eight persons were given, one at a time, 10 pairs of twigs to peel (feed tree and nonfeed tree) and asked to choose between two alternatives: a difference or no difference in the ease of peeling and separating inner and outer bark between the pair of twigs. Twig pairs were labeled randomly with letters or numbers to aid in identifying twigs from feed or nonfeed trees after responses had been made. Total responses would equal 640 (10 pairs/person; 8 persons/sampling date; 8 sampling dates).

Data were subjected to $t$ tests with unpaired observations comparing feed-tree inner–outer bark with nonfeed–tree inner–outer bark and inner bark to outer bark. For the ease of peeling study, a $t$ test with unpaired observations was used to compare between feed and nonfeed trees.

## RESULTS

Monoterpenoid content of outer bark for individual nonfeed trees over the study period ranged from 0.50 to 1.19% of dry matter. The overall mean was 0.75% with a standard deviation of 0.19%. For feed trees, the monoterpenoid

[3] The use of trade, firm, or corporation names is for information and convenience of the reader. Such does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others which may be suitable.

content ranged from 0.31 to 1.87%. Overall mean was 0.77% with a standard deviation of 0.29%. Monoterpenoid content for feed-tree outer bark was not significantly higher than for nonfeed trees (Table 1).

Monoterpenoid content of inner bark for individual nonfeed trees over the study period ranged from 0.00 to 1.38% of dry matter. Overall mean was 0.16% with a standard deviation of 0.17%. For feed trees, the monoterpenoid content ranged from 0 to 1%. Overall mean was 0.10% with a standard deviation of 0.10%. Monoterpenoid content for nonfeed-tree inner bark was not significantly higher than for feed trees (Table 1). Pooling feed and nonfeed trees, outer bark (0.76%) contained significantly higher concentrations of monoterpenoids than inner bark (0.13%).

On an individual monoterpenoid basis, four monoterpenoids were identified in outer bark and only one in inner bark. Outer bark contained $\alpha$-pinene, $\beta$-pinene, (+)-3-carene, and 1,8-cineol. $\alpha$-Pinene was the only monoterpenoid in inner bark.

$\alpha$-Pinene content of outer bark for individual nonfeed trees over the study period ranged from 0.28 to 1.10% of dry matter. The overall mean was 0.61% with a standard deviation of 0.23%. For feed trees, $\alpha$-pinene content ranged from 0.23 to 0.78% of dry matter. Overall mean was 0.51% with a standard deviation of 0.15%. $\alpha$-Pinene content for nonfeed-tree outer bark was not significantly higher than for feed trees. Outer bark contents for $\beta$-pinene, (+)-3-carene, and 1,8 cineol are as follows: $\beta$-pinene $-0.05 \pm 0.06\%$ nonfeed trees, $0.12 \pm 0.14\%$ feed trees; (+)-3-carene, $0.08 \pm 0.07\%$ nonfeed trees, $0.14 \pm 0.16\%$ feed trees; and 1,8-cineol, $0.01 \pm 0.05\%$ nonfeed trees, $0.05 \pm 0.09$ feed trees. $\beta$-Pinene, (+)-3-carene, and 1,8-cineol contents for feed trees were not significantly higher than for nonfeed trees.

Crude protein content of outer bark for individual nonfeed trees over the study period ranged from 1.9 to 2.9% of dry matter. The overall mean was 2.3% with a standard deviation 0.3%. For feed trees, the crude protein content ranged from 2.0 to 3.1% of dry matter. Overall mean was 2.4% with a standard deviation 0.3%. Feed trees did not contain significantly higher levels of crude protein than nonfeed trees.

Crude protein content of inner bark for individual nonfeed trees over the study period ranged from 2.1 to 3.2% of dry matter. The overall mean was 2.4% with a standard deviation of 0.3%. For feed trees inner bark crude protein content ranged from 1.9 to 3.0% of dry matter. Overall mean was 2.4% with a standard deviation of 0.2%.

The remaining components of proximal analysis—crude fiber, crude fat, ash, and nitrogen-free extract—were found not to be significantly different between nonfeed and feed trees. However, inner bark contained significantly higher levels of ash (5.8% vs. 2.4% dry matter) than outer bark but significantly lower levels of crude fat (3.2% vs. 11.6% of dry matter). Crude fiber content for both bark tissues was 10.3% of dry matter and 61.5% of dry matter for nitrogen free extract.

TABLE 1. TOTAL MONOTERPENOID CONTENT OF OUTER AND INNER BARK FROM PONDEROSA PINE TREES FED AND NOT FED UPON BY ABERT SQUIRRELS[a]

| Type of bark | Tree type | Months | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | April | May | July | August | October | December | January | March | May |
| Outer bark | Feed trees | $0.77 \pm 0.76$ | $0.64 \pm 0.27$ | $0.66 \pm 0.13$ | $0.74 \pm 0.16$ | $0.87 \pm 0.16$ | $0.71 \pm 0.12$ | $1.08 \pm 0.63$ | $0.72 \pm 0.15$ | $0.76 \pm 0.24$ |
| | Nonfeed trees | $0.87 \pm 0.20$ | $0.74 \pm 0.16$ | $0.75 \pm 0.15$ | $0.83 \pm 0.17$ | $0.73 \pm 0.17$ | $0.72 \pm 0.13$ | $0.72 \pm 0.17$ | $0.77 \pm 0.12$ | $0.64 \pm 0.40$ |
| Inner bark | Feed trees | $0.05 \pm 0.02$ | $0.05 \pm 0.03$ | $0.05 \pm 0.04$ | $0.10 \pm 0.05$ | $0.03 \pm 0.03$ | $0.03 \pm 0.04$ | $0.32 \pm 0.39$ | $0.15 \pm 0.15$ | $0.13 \pm 0.12$ |
| | Nonfeed trees | $0.10 \pm 0.07$ | $0.11 \pm 0.08$ | $0.11 \pm 0.07$ | $0.11 \pm 0.07$ | $0.08 \pm 0.08$ | $0.11 \pm 0.10$ | $0.39 \pm 0.56$ | $0.23 \pm 0.27$ | $0.16 \pm 0.22$ |

[a]Data expressed as a percentage of dry matter; means and standard deviations based on five replications (1980–1981).

Results from the ease of peeling test were quite remarkable. Out of 640 responses, all 640 were the same. Bark from feed–tree twigs was easier to peel and to separate into inner and outer bark than bark from nonfeed–tree twigs.

## DISCUSSION

In contrast to the finding of Farentinos et al. (1981), we found no significant difference in total monoterpenoid levels between nonfeed and feed trees for inner bark. There are two major differences in the way Farentinos et al. (1981) and our studies were conducted. First, their analyses were conducted on inner bark tissue, whereas we ran analyses on both outer and inner bark. Our reasoning was that the squirrel first had to chew through the outer bark before consuming the inner bark tissues. Thus, factors that influence tree selectivity could occur in the outer bark and not in the underlying inner bark. Secondly, Farentinos et al. (1981) used a steam-distillate with *n*-pentane and expressed their data in terms relative to an internal standard or as a percent composition of steam-distillate with *n*-pentane. Our study used solvent extraction, and we expressed our data on a dry matter basis. In our study there appears to be a trend of lower monoterpenoid content in the inner bark tissue of feed trees than for nonfeed trees. But due to large variation (expressed as standard deviations in Table 1) among individual trees, statistical tests used were unable to detect differences. Farentinos et al. (1981) also reported large standard deviations (standard deviations larger than 50% of the mean). Others working with wild animals have reported no relation between food preference and monoterpenoids (white-tailed deer, Connolly et al., 1980; black-tailed deer, Radwan and Crouch, 1978; mule deer, Scholl et al., 1977; pygmy rabbits, White et al., 1982; Welch et al., 1983). However, Schwartz et al. (1980), using tame mule deer in a cafeteria feeding trial, reported a significant relationship between mule deer food preference and monoterpenoid content. Remington (1983) noted that sage grouse preferred Wyoming big sagebrush (*Artemisia tridentata* ssp. *wyomingensis*), which is lower in monoterpenoids, over mountain big sagebrush (*A. t.* ssp *vaseyana*). Within Wyoming big sagebrush, monoterpenoid content was not related to feed or nonfeed plants.

The significance of the outer bark containing higher levels of monoterpenoids than the inner bark in relation to food selection is not clear. Inner bark is what is eaten by the squirrels, but squirrels must first chew through the outer bark. Because total and individual monoterpenoid content for inner and outer bark of both feed and nonfeed trees was not statistically different, it is questionable that monoterpenoids play a role in the selection of feed or nonfeed trees, but they could play a major role in the part of the bark eaten.

In our opinion, the major determinant in feed tree selection is the ease of bark peeling and of separating outer bark from inner bark.

One last point from our study concerns the low crude protein content of the inner bark (2.4% ± 0.2%). This low level of an essential nutrient explains the observations made by Stephenson and Brown (1980) that mortality and reproduction of Abert squirrels were related to the number of days with snow depth of 10 cm or more. During these days, foods having a higher crude protein content are covered with snow, forcing the squirrels to feed entirely on inner bark of ponderosa pine. The minimum daily protein requirement for laboratory rodents is 15% crude protein (Bierl et al., 1977). Protein deficiency is the probable cause of high winter mortality and reduced productivity during winters with deep and lasting snow cover.

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# IDENTIFICATION OF SEX PHEROMONE COMPONENTS OF JACK PINE BUDWORM, *Choristoneura pinus pinus* FREEMAN[1]

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**Abstract**—Chemical identification and field-trapping experiments have shown that a blend of 85:15 (*E,Z*)-11-tetradecenyl acetates and 85:15 (*E,Z*)-11-tetradecen-1-ols (in a 9:1 ratio) are female sex pheromone components for jack pine budworm, *Choristoneura pinus pinus*. This blend of chemicals, formulated in PVC (0.1%, w/w) sources is as effective a trap bait as virgin females. Preliminary wind tunnel observations have indicated that this blend, effective as a trap bait, is not equivalent to females.

**Key Words**—*Choristoneura pinus pinus*, jack pine budworm, Lepidoptera, Tortricidae, (*E,Z*)-11-tetradecenyl acetate, (*E,Z*)-11-tetradecen-1-ol, sex pheromone, sex attractant.

## INTRODUCTION

The coniferophagous budworms comprise a group of closely related (*Choristoneura*) species native to North America (Freeman, 1967). Two economically important eastern species, the spruce budworm *Choristoneura fumiferana* (Clem-

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ens) and the jack pine budworm *Choristoneura pinus pinus* Freeman, are sympatric for part of their ranges in the Acadian, Great Lakes–St. Lawrence and boreal forest types (Freeman, 1953; Stehr, 1967). *C. fumiferana* feeds primarily on *Abies* and *Picea* spp. (rarely on *Pinus* spp.), while *C. pinus pinus* feeds primarily on *Pinus* spp. notably *P. banksiana* and *P. resinosa* (Freeman and Stehr, 1967).

Although the potential for hybridization of these two moth species is reduced by temporal isolation and spatial separation due to host distribution (Sanders, 1971a), the ultimate barrier would appear to be the specificity of their respective sex pheromones since neither is cross-stimulatory or cross-attractive (Sanders, 1971b). In attempting to separate species through cross-attraction studies, Sanders et al. (1977) concluded that *C. fumiferana, C. occidentalis*, and *C. biennis* apparently have similar pheromones, while *C. pinus pinus, C. orae*, and *C. retiniana* (= *C. viridis*) are mutually cross-stimulating but do not appear to share sex pheromone components with the former group. Sex pheromone components of several of these species have recently been documented: *C. fumiferana* (Sanders and Weatherston, 1976; Silk et al., 1980) and *C. occidentalis* (Cory et al., 1982; Silk et al., 1982) have been shown to release *(E,Z)*-11-tetradecenal (*E,Z* 11–14 : Ald) as primary sex pheromone components whereas *C. retiniana* (Daterman et al., 1984) and *C. orae* (Gray et al., 1984) release *(E,Z)*-11-tetradecenyl acetates (*E,Z* 11–14 : Ac) as primary sex pheromone components. Additional (secondary) components have recently been identified from two of these species (*C. fumiferana*, Alford et al., 1983; and *C. occidentalis*, Alford and Silk, 1983).

This study was undertaken to isolate and identify pheromone components of *C. pinus pinus*, the jack pine budworm, which is a serious defoliator in Ontario (Applejohn and Howse, 1983), Manitoba, Saskatchewan, Quebec, and the lake states of the United States (Prebble, 1975). We report here that 85 : 15 *E,Z* 11–14 : Acs and 85 : 15 *E,Z* 11–14 : OHs are released by female jack pine budworm. Traps baited with a 90 : 10 blend of these compounds in polyvinyl chloride (0.1% w/w) captured as many males as did virgin female-baited traps.

## METHODS AND MATERIALS

*Insects.* Jack pine budworm pupae were collected in Blackville, N.B. from several jack pine stands between 1981 and 1983. Several collections near Kirkwood, Ontario were also made during this period. Pupae were separated by sex and held for emergence at *ca.* 25°C on a 17:7 L:D cycle.

*Collection of Pheromone.* Sex pheromone glands were excised from 1- to 4-day-old virgin females, during their pheromone release period, 1–3 hr into scotophase (Sanders, 1971a). Excised glands were soaked overnight in freshly distilled methylene chloride (0.5 ml) in groups of 20–60. Gland extracts obtained

in this way were purified by silica gel column chromatography (Silk et al., 1980) and redissolved in hexane prior to analysis by gas chromatography (GC).

Effluvial volatiles from females were collected in two ways. Firstly, volatiles were obtained by collecting pheromone volatilized from groups of females using a Porapak® Q technique previously developed for *C. fumiferana* (Silk et al., 1980). Secondly, effluvia from single insects which were 2–3 hr into scotophase were collected onto glass wool (Baker et al., 1981) and eluted with hexane. Both collections of volatiles were analyzed by capillary GC and GC–mass spectrometry (MS).

*Electroantennogram (EAG) Analysis.* GC fractionated material, silica gel column fractions, and synthetic standards were used as stimuli for evaluation of EAG responsiveness (Roelofs, 1977; Silk et al., 1982); when synthetics were tested, 10-ng source concentrations were used ($N = 5$).

*Instrumental Analysis.* GC analyses were performed on capillary columns on a Varian 3700 gas chromatograph with a flame ionization detector; hydrogen was used as the carrier gas at an inlet pressure of 0.5 kg/cm$^2$. A 40-m SP1000 0.5-mm-ID, SCOT capillary column (SGE) was used; injections were made in the splitless mode. GC-MS was performed on a Finnigan 4021 EI-CI-INCOS system using the same SCOT column. Helium was used as the carrier gas with injections made in the splitless mode and the column at ambient temperature. The splitter was opened 30 sec after injection, and then the column was temperature programed at 30°/min to 150°C and 8°/min to 190°C. GC-MS conditions were such that ca. 5 ng of synthetic $\Delta$ 11-$C_{14}$ acetates, alcohols, and aldehydes produced excellent full-scan mass spectra and resolution of all respective *E* and *Z* isomers.

Identified peaks were quantitated by comparison of peak heights with those from internal and external standards. Analyses were replicated and corrections made for procedural recoveries. Appropriate controls from collection procedures showed no interfering peaks on capillary GC. Components were identified by retention time on capillary column GC and by their electron impact–chemical ionization [EI-CI (methane)] mass spectra compared with authentic synthetic material. Double bond positions were determined by microozonolysis of extracts and standards, using a syringe type ozonizer (Beroza and Bierl, 1967); ozonides were cleaved with dimethyl sulfide and the products analyzed by GC-MS.

*Chemicals.* Chemicals were obtained from Chemical Samples Company, Columbus, Ohio. They were ≥98% pure as determined by capillary GC analysis and were used without further purification.

*Field Bioassays.* In 1983, following identification of possible pheromone components and preliminary wind tunnel observations, appropriate synthetic blends were formulated into polyvinyl chloride (PVC) rods or in polyethylene vial-caps for use as trap baits. Nominal release rates of these trap baits were measured using a modified single-insect collection device described above.

Trapping experiments were conducted in Blackville, New Brunswick, in a

mixed stand of jack pine and balsam fir which had noticeable defoliation of both species, and in Kirkwood, Ontario, in a jack pine plantation. In New Brunswick, traps (Pherocon®-1C) were placed out in a randomized complete block design (3 blocks) from July 9 to 30. Traps were checked, trap bottoms changed, and the treatments rerandomized every three to seven nights. In Ontario, traps (Pherocon-1CP) were placed out in randomized complete blocks from July 15 to 22 in three separate areas (all within 1 km). Traps were checked on July 18, 20, and 22, and on the first two dates each trap was moved ahead one position in the grid. Virgin female-baited traps were included for comparison at the Ontario site. Traps in all plots were ca. 20 m apart, 1.5 m from the ground. Trap capture data (mean number of moths/trap/night at each check date) were analyzed by two-way analysis of variance, and log-transformed means (following Bartlett's test for homogeneity of variances) were separated by Duncan's new multiple-range test.

## RESULTS AND DISCUSSION

*Chemical Analysis.* Virgin female gland extracts contained predominantly *E,Z* 11–14:Ac ($\bar{X}$= 26.5 ng/gland; range 6.7–64.6). Although considerable variability in total acetate existed, the determined *E,Z* ratio in each extract showed only minor variability with the *Z* isomer at 13–15% of the *E* isomer. *E,Z* 11–14:OHs (*E,Z* 85:15) were also present ($\bar{X}$ = 3.0 ng/gland; range 0.2–7.0) along with the saturated analogs 14:Ac and 14:OH, which were present at 2–3% of their respective *E* unsaturated analogs. No corresponding aldehyde components were detected. Separation of *E,Z* isomers for both the acetate and alcohol components, was effected using the SP1000 capillary column; respective *E* and *Z* isomers of all insect-derived components had identical retention times and EI-CI mass spectra compared with authentic synthetic material. Ozonolysis of gland extracts and synthetic standards followed by capillary GC-MS analyses verified that the double bond position in both the unsaturated acetates and alcohols was $\Delta$11 since $\Delta$11-acetoxy undecanal and 1-hydroxy undecanal were produced.

Analyses of effluvial extracts obtained using *Porapak Q collection* showed that *E,Z* 11–14:OHs were the major components ($\bar{X}$ = 16 ng/female-night: range 7.9–45.3). In each determination, only small amounts of *E,Z* 11–14:Acs (*E,Z* 85:15) were detected (ca. 1% of *E* 11–14:OH).

*Single Insect Collection.* Volatiles from single insects collected on glass wool produced a totally different GC profile. In this case, *E,Z* 11–14:Acs were the predominant components (ca. 1–4 ng/hr; *E,Z,* 85:15) with *E,Z* 11–14:OH at only 5–15% of *E* 11–14:Ac. A dichotomy, therefore, existed between the acetate–alcohol ratio determined by these two techniques of collection. Field-trapping assays concentrated primarily on *E,Z* 11–14:Ac and *E,Z* 11–14:OH as candidate components.

*Electroantennograms.* Of all the synthetics tested with EAGs only the acetates elicited strong antennal responses with *E* 11–14:Ac (2.0 mV) giving the largest depolarizations (all responses were normalized to a 10-ng source of 85:15 *E,Z* 11–14:Ac; mean 2.1 mV, range 1.2–4.0 mV). Alcohols, including 85:15 *E,Z* 11–14:OHs (0.3 mV) gave weak responses. Bornyl acetate, a known constituent of jack pine foliage gave a moderate response (1.1 mV). The 8% diethyl ether–hexane fractions, obtained from silica gel chromatography of either glandular or effluvial material, elicited consistently strong EAG responses. This fraction would contain the *E,Z* 11–14:Acs (Silk et al., 1980). Glandular and effluvial material was fractionated by GC, and consecutive samples were collected in cooled (Dry Ice-in-acetone bath) glass capillaries. EAG responses resulted only from samples having retention times corresponding to *E,Z* 11–14:Acs.

From chemical analyses and EAG data, we concluded that the sex pheromone of *C. pinus pinus* might involve *E,Z* 11–14:Ac and possibly, *E,Z* 11–14:OH.

*Field Testing.* In 1981 and 1982, traps were baited primarily with sources (PVC and vial-caps) loaded with 85:15 *E,Z* 11–14:OH with admixtures of 0–50% 85:15 *E,Z* 11–14:Ac. Few males were captured. These blend ratios were predicated on the Porapak Q collection data.

Preliminary wind tunnel observations (Kuenen and Silk, unpublished data) of males responding to "calling" females, to everted female glands, and to synthetics indicated that none of the synthetics that were field tested elicited upwind flight and arrival at the source; females or extruded pheromone glands almost always elicited upwind male flight and arrival at the source. Upwind flight did not occur when synthetic 85:15 *E,Z* 11–14:Acs or 85:15 *E,Z* 11–14:OHs alone were tested, although the acetates elicited a male wing-fanning response in almost every case. As noted above, the single-insect collections subsequently showed that 85:15 *E,Z* 11–14:Acs were the major components volatilizing from extruded pheromone glands, with 5–15% 85:15 *E,Z* 11–14:OHs also present. In the flight tunnel, sources with blends approximating these ratios elicited a marked increase in male activation and upwind flight compared with their responses to the alcohol-rich sources; none of the blends tested were equivalent to females.

In 1983, field tests were conducted with synthetic sources containing predominantly the acetate components. The blend containing 90% 85:15 *E,Z* 11–14:Ac and 10% 85:15 *E,Z* 11–14:OH (0.1% w/w PVC) captured the most males in both New Brunswick and Ontario (Tables 1 and 2). This formulated blend was also at least as good as virgin females (2/trap) in capturing males over several days in the field (Table 2), and its release rate and acetate–alcohol ratio, determined with the single insect collection device, was ca. 1 ng/hr (acetate) and ca. 0.18 ng/hr (alcohol), giving a release rate and blend ratio close to that from virgin females. Neither the acetate nor the alcohol components alone were active in the field, as expected from the wind tunnel observations.

TABLE 1. NEW BRUNSWICK TRAP CAPTURE OF *C. pinus pinus* MALES IN PHEROCON-IC TRAPS

| Treatment[a] | Formulation | Mean No. moths/trap/night[b] |
|---|---|---|
| 90% A + 10% B | 0.1% PVC | 9.3 a |
| 95% A + 5% B | 1.0% PVC | 7.8 ab |
| 100 μg A + 10 μg B | cap | 6.7 ab |
| 80% A + 20% B | 0.1% PVC | 6.7 ab |
| 90% A + 10% B | 1.0% PVC | 6.4 ab |
| 500 μg A + 500 μg B | cap | 5.1 bc |
| 80% A + 20% B | 1.0% PVC | 4.1 c |
| 100 μg A + 100 μg B | cap | 4.1 c |
| 95% A + 5% B | 0.1% PVC | 3.8 c |
| 99% A + 1% B | 1.0% PVC | 1.4 d |
| 50% A + 50% B | 1.0% PVC | 1.3 d |
| 99% A + 1% B | 0.1% PVC | 1.2 d |
| 50% A + 50% B | 0.1% PVC | 0.9 de |
| 100 μg A | cap | 0.7 de |
| 100% A | 0.1% PVC | 0.5 ef |
| 100% B | 0.1% PVC | 0.5 ef |
| Control (empty trap) | | 0.5 ef |
| 500 μg A | cap | 0.4 ef |
| 100% A | 1.0% PVC | 0.3 f |
| 100% B | 1.0% PVC | 0.2 f |

[a] A = 85:15 *E,Z*11-14:Ac; B = 85:15 *E,Z*11-14:OH.
[b] Antilog$_{10}$($X$)-1, of log$_{10}$ (mean of block means); means having no letters in common are significantly different, DNMRT, $P < 0.05$.

## CONCLUSIONS

Our laboratory and field trapping studies indicate that a PVC rod containing 0.1% "pheromone" by weight (90% 85:15 *E,Z* 11–14:Ac + 10% 85:15 *E,Z* 11–14:OH) was as effective at trapping males as were virgin females. This sex attractant formulation could be used at this time for detection and monitoring of jack pine budworm. However, our preliminary observations in a sustained-flight wind tunnel indicate that these compounds in this particular blend and release rate do not represent the complete pheromone blend. It is not known if blend and release rate changes or the addition of other (unidentified) pheromone components would enhance trap capture; work is in progress in this area. A better definition of a complete pheromone blend becomes much more important with the prospect of using pheromones for control procedures (Roelofs, 1978; Silk and Kuenen, 1984) since the "complete" pheromone would likely be necessary for implementation of male annihilation techniques and may also be important for mating disruption (e.g., Charlton and Cardé, 1981).

TABLE 2. ONTARIO TRAP CAPTURE OF *C. pinus pinus* MALES IN PHEROCON-ICP TRAPS

| Treatment[a] | Formulation | Mean No. moths/trap/night[b] |
|---|---|---|
| 90% A + 10% B | 0.1% PVC | 9.9 a |
| 100 μg A + 10 μg B | cap | 5.5 ab |
| 80% A + 20% B | 0.1% PVC | 5.2 ab |
| 95% A + 5% B | 0.1% PVC | 5.1 ab |
| Virgin females (2/trap) | | 4.8 ab |
| 50% A + 50% B | 0.1% PVC | 4.5 ab |
| 90% A + 10% B | 1.0% PVC | 4.1 abc |
| 500 μg A + 50 μg B | cap | 3.9 abc |
| 95% A + 5% B | 1.0% PVC | 3.2 bcd |
| 99% A + 1% B | 0.1% PVC | 2.8 bcde |
| 80% A + 20% B | 1.0% PVC | 2.1 bcdef |
| 500 μg A + 500 μg B | cap | 1.9 bcdef |
| 50% A + 50% B | 1.0% PVC | 1.0 cdefg |
| 100% B | 0.1% PVC | 1.1 cdefg |
| 100 μg A + 100 μg B | cap | 1.1 cdefg |
| 99% A + 1% B | 1.0% PVC | 0.7 defg |
| 100% B | 1.0% PVC | 0.6 efg |
| 100% A | 0.1% PVC | 0.4 fg |
| 100 μg A | cap | 0.3 fg |
| 100% A | 1.0% PVC | 0.05 g |
| 500 μg A | cap | 0.05 g |

[a]A = 85 : 15 *E,Z*11-14 : Ac; B = 85 : 15 *E,Z*11-14 : OH.
[b]Antilog$_{10}$($X$)-1, of log$_{10}$ (mean of block means); means having no letters in common are significantly different, DNMRT, $P < 0.05$.

The cause(s) of the dichotomy that exists between data obtained from volatiles collected with Porapak Q compared with collections from single insects on glass wool should be examined. Clearly, in the case of the unsaturated acetate-alcohol mixtures identified in *C. pinus pinus*, the Porapak Q technique yielded the "wrong" acetate-alcohol ratio. However, recoveries of unsaturated $C_{14}$ acetates and alcohols using the Porapak Q technique are reproducible and quantitative under control conditions (Silk et al., 1980). This strongly implies that the presence of living insects causes the "wrong" acetate-alcohol ratio. When *C. pinus pinus* females are incubated for 5 hr in a 50-ml flask with 1 or 10 μg *E* 11-14 : Ac, at least 50% is recovered as *E* 11-14 : OH (as determined by GC) compared to complete acetate recovery with no females in the flasks (Silk et al., unpublished results). Since degradation of pheromone components by external, cuticle-borne enzymes is known (Ferkovich et al., 1982), it appears likely that our "wrong" acetate-alcohol ratio and recovery of alcohol from insect-incubated acetate resulted from hydrolysis of acetate to alcohol by enzymes or other factors found on *C. pinus pinus* females. Our results, therefore, raise

a cautionary note regarding the use of the Porapak Q technique for determination of pheromone components and ratios.

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# SOME VOLATILE CONSTITUENTS OF FEMALE DOG URINE

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**Abstract**—The volatile compounds from female beagle urine, across the state of estrus, were examined by headspace gas chromatography and mass spectrometry. The major constituents identified were methyl propyl sulfide, methyl butyl sulfide, and acetone. Nine minor constituents, including trimethyl amine and five disulfides, were identified. Two of the unidentified minor constituents may possibly be associated with the state of estrus.

**Key Words**—Canidae, dog urine, volatiles, estrus, methyl propyl sulfide, methyl butyl sulfide, acetone.

## INTRODUCTION

There has been considerable interest in the composition of the vapors from secretions of canids, which play a part in olfactory communication among members of this family (Preti et al., 1976; Teranishi et al., 1981; Murphy et al., 1978). A particular question involves volatile compounds present in secretions of females during the period of estrus (Jorgenson et al., 1978; Goodwin et al., 1979). The attractancy of female beagle estrous urine to male beagles is pronounced (Doty and Dunbar, 1974). However, during 24 hr of storage of the urine in a loosely capped, clear-glass bottle at room temperature, the strength of this stimulus decreased to that of fresh anestrous urine (McKenna, 1981). This suggests that the key attractive compound, or compounds, are highly volatile or chemically labile.

This paper reports on the identification of some of the volatile constituents of female beagle urine across the state of estrus. The work is part of a broader

study which includes the coyote. Such information is desired because it may be helpful in improving control of feral dogs and coyotes and thus reducing predation losses of livestock. The analyses were run on vapors from the headspace of fresh urine samples at room temperature. Gas chromatography (GC) and the same combined with mass spectrometry (GC-MS) were employed, with relatively mild conditions throughout the procedure, in the hope that neither highly volatile constituents nor labile compounds would be lost.

## METHODS AND MATERIALS

*Collection of Urine Samples.* Four female beagles, mean age 5 years, that had had pups in previous seasons were housed together in an outdoor five-acre pen and maintained on a standard diet of commercial dry kibble and water. None of their estrous periods overlapped. As each dog approached proestrus, she was moved to a separate kennel and kept alone until she became anestrous again. Dogs 1 and 2 were sampled irregularly. With dogs 3 and 4, samples were taken at intervals of two or three days when possible. Thirteen samples in all were taken. On the night before a urine sample was to be collected, the dog was placed in a metabolism cage in a thoroughly cleaned testing room which had a polyamine–epoxy-coated floor. The floor had just been cleaned again with ammonia and thoroughly rinsed with water. (Usually, no urine was donated in the cage.) On the following morning, the dog usually donated a urine sample onto the floor soon after being released from the cage. The urine was drawn into a disposable syringe within 30 sec of donation and collected in a glass bottle. This was kept in an ice bath for about 2 hr while delivering it to the laboratory and starting the analysis.

The estrous state of the animal was determined by vaginal cytology on a smear taken within 4 hr after the urine sample was taken.

*Headspace Analysis.* The method used comprised sweeping the surface of the urine sample with nitrogen, directing this carrier gas with the urine vapors through a small trap packed with an adsorbent, back-flushing the adsorbed volatiles into a cold trap, flashing the volatiles into a GC column, and carrying out the GC run as usual (Schultz et al., 1971). The apparatus described in this cited paper was modified by adding another switching valve between the adsorbent trap (precolumn) in its second location and the spiral trap (cold trap), and rearranging the units to enable more facile and direct operation.

The adsorbent traps used were 3-in. lengths of 0.25-in. OD stainless-steel tubing with fritted disks pressed in the lower ends and Swagelok[3] fittings at both ends. Each of these traps was packed with ca. 0.19 g of 60–80 mesh Tenax GC

[3] Reference to a company and/or product named by the department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

(Applied Science Laboratories, Inc., State College, Pennsylvania 16801) (Noble et al., 1980).

The urine sample (12–50 ml) in the 250-ml Erlenmeyer flask, with magnetic stirring, was swept 2 hr with nitrogen at 16 ml/min at room temperature. Flow through the Tenax trap was upward. A 12-in. × 0.03-in. ID stainless-steel coiled tube served as a tailpiece to prevent back diffusion of atmospheric contaminants. After the sweeping period, the nitrogen delivery tube was coupled directly to the Tenax trap and flow was continued 15 min to carry out most of the remaining water. Then the Tenax trap was moved to the back-flushing position and the procedure continued without delay, or it was plugged at both ends and kept at −18°C until final analysis.

Back-flushing was accomplished with helium flowing downward at 32 ml/min for ca. 12 min during the warm-up period and for an additional 30 min at 125–128°C. A Dewar flask of liquid nitrogen was used for cooling the spiral trap and a beaker of glycerol at 140°C was used for heating it.

Immediately at the close of back-flushing, the Tenax trap was heated further, to 175°C for 30 min, with an increased flow of helium, at 120 ml/min, in order to thoroughly clean it before using it again.

*GC Conditions.* The GC column was one of dual 500-ft, 0.03-in. ID, open-tubular stainless-steel columns coated with methyl silicone oil SF 96(50) (General Electric, Waterford, New York) mixed with 5% of Igepal CO-880 (General Aniline and Film Corp., New York, New York). Dual hydrogen-flame ionization detectors were employed. The helium-head pressure was 10 psig, which gave a front time of 6.8 min. Column temperature was held at 60°C for the first 10 min and then was programed at 1.67°/min to 175°C, where it was held constant for the remainder of the run. An automation system (model 3385A, Hewlett-Packard, Palo Alto, California 94304) was employed to plot the chromatogram and automatically report the areas of the peaks of interest.

*GC-MS.* A modified quadrupole mass spectrometer system described by Noble et al. (1980) was used. However, the same GC column and operating conditions described above were used. The interface between the GC column exit and the mass spectrometer was a molecular separator with a silicone rubber membrane (Forrey and Flath, 1974), held at 180–185°C. Mass spectra were recorded by a three-channel oscillograph on single 1-sec scans. Only one GC-MS run was made on volatiles trapped from the urine sample collected on June 6 from dog 4.

## RESULTS AND DISCUSSION

A list of the identified constituents of the headspace vapor of female beagle urine is shown in Table 1. All of the compounds, except the unsymmetrical disulfides, were identified by both their mass spectra matched with literature spectra of known compounds and GC retention behavior. The unsymmetrical

TABLE 1. FEMALE BEAGLE URINE VOLATILE CONSTITUENTS

| Peak Number[a] | Compound | Relative amount |
|---|---|---|
| 3 | Trimethyl amine | Minor |
| 5 | Acetone | Major |
| 6 | Ethanol | |
| 11 | 2-Butanone | Minor |
| 17 | 3-Methylbutanal | Minor |
| 19 | Benzene | Doubtful[b] |
| 25 | Methyl propyl sulfide | Major |
| 27 | Dimethyl disulfide | Minor |
| 29 | Toluene | Doubtful[b] |
| 33 | Methyl butyl sulfide | Major |
| 44 | Methyl "pentenyl"[c] sulfide | Minor |
| 48 | Methyl propyl disulfide[d] | Minor |
| 59 | Methyl butyl disulfide[d] | Minor |
| 68 | Dipropyl disulfide | Minor |
| 73 | Propyl butyl disulfide[d] | Minor |

[a]Peak numbers refer to the chromatogram in Figure 1.
[b]May be an artifact.
[c]"Pentenyl": A monounsaturated, branched, primary C-5 group. Five isomers are possible, the one which is present is as yet unknown.
[d]Tentative.

disulfides were suggested by interpretation of their mass spectra only, without any reference compounds or published spectra; thus these identifications are very tentative. Peak numbers in the first column refer to the GC chromatogram shown in Figure 1. This figure is a hand-drawn tracing of the instrument-made plot, with omission of peaks representing atmospheric contaminants, which were found in blank runs.

The major constituents were methyl propyl sulfide (peak 25), methyl butyl sulfide (peak 33), and acetone (peak 5). These were consistently prominent constituents in all samples from all four of the dogs. (Peak 6 was usually not resolved from peak 5.) Several of the minor constituents are disulfides. Peaks for benzene and toluene were observed in blank runs also, but they appear considerably larger in some of the runs on the urine samples. However, it is not clear whether these aromatic hydrocarbons are urine constituents or not.

Several of the compounds listed in Table 1 have been found in secretions of other canid species. Trimethyl amine has been reported to be a constituent of the anal sac secretions of the female red fox (*Vulpes vulpes fulva* L.) (Albone and Fox, 1971), and both the male and female dog (*Canis familiaris*) and coyote (*Canis latrans*) (Preti et al. 1976). The major sulfur compounds, methyl propyl sulfide and methyl butyl sulfide (and dimethyl disulfide) have been reported to be constituents of the urine of the coyote (Teranishi et al., 1981), but not as

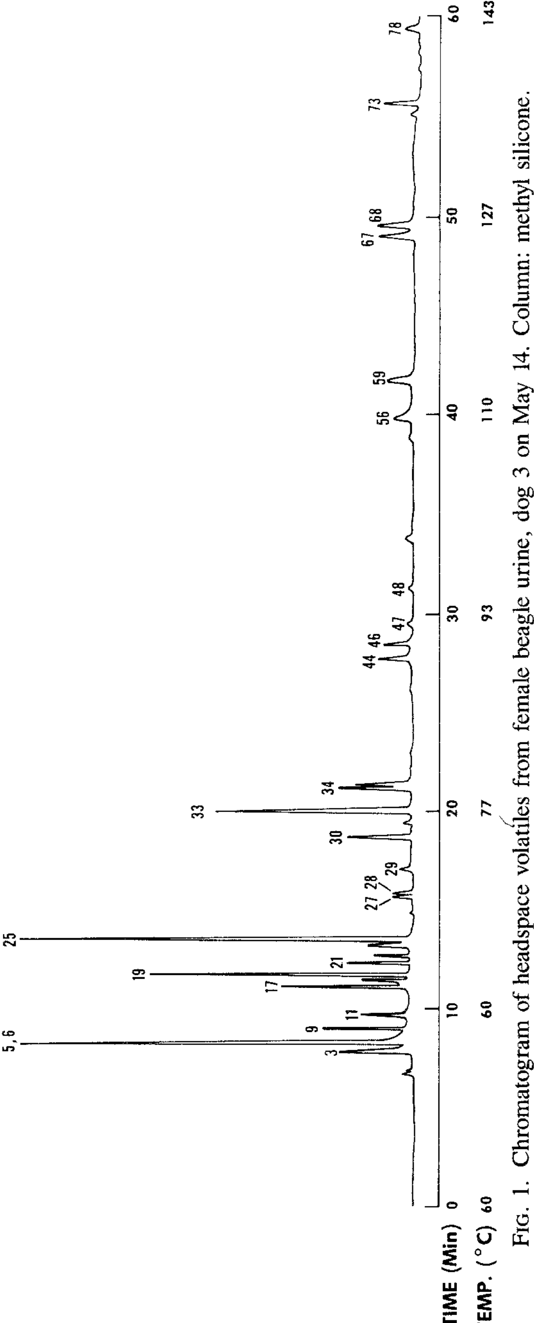


FIG. 1. Chromatogram of headspace volatiles from female beagle urine, dog 3 on May 14. Column: methyl silicone.

major constituents. Methyl 3-methylbut-3-enyl sulfide (Δ3-isopentenyl methyl sulfide) has been reported to be a constituent of the urine of the red fox (*Vulpes vulpes* L.) during the winter season (Jorgenson et al., 1978). The methyl "pentenyl" sulfide listed in Table 1 (peak 44) is this same compound or one of its isomers.

No information was obtained about the possible presence of carboxylic acids, phenolic compounds, or mercaptans in the urine headspace because the GC column used does not permit the passage of small amounts of these compounds as definite peaks.

Relative concentrations of four of the urine constituents across the state of estrus for dog 4 are shown in Table 2. The major sulfides appear at somewhat higher concentrations at the proestrous and estrous periods than at the metestrous or anestrous, but this may be due to normal variations not related to estrus. A search of the data was made to find peaks which were present at the estrous period but absent, or lower by at least one order of magnitude, at the anestrous. No definite example was found, except perhaps peak 47 (unidentified). The highest concentration (1200) of this constituent was observed at the beginning of full estrus of dog 4, and peak areas at metestrus and anestrous were well below 100. However, agreement between dogs 3 and 4 was only fair, and peak areas in the single runs on dogs 1 and 2, both at estrus, were quite low. Peak 46 (unidentified) also is interesting, but its highest concentration was observed at metestrus of dog 4. Perhaps the data might be more consistent if many more samples were taken at daily intervals. These results suggest that the substances

TABLE 2. FEMALE BEAGLE URINE VOLATILES: GC PEAK AREAS[a]

| Sample date | State of estrus | Peak number and compound | | | |
|---|---|---|---|---|---|
| | | 25 Me Pr S | 33 Me Bu S | 46 Unidentified | 47 Unidentified |
| Dog. 3 | | | | | |
| 5-14 | Estrous | 4000 | 1700 | 270 | ~60 |
| 5-16 | Estrous | [b] | 1200 | ~30 | 540 |
| 5-19 | Estrous | 7500 | 1300 | ~50 | 300 |
| 5-21 | Estrous | 5100 | 1000 | ~20 | 500 |
| Dog 4 | | | | | |
| 5-31 | Proestrous | 5700 | 3700 | 40 | 200 |
| 6-3 | Borderline | 5000 | 4400 | ~20 | 1200 |
| 6-5 | Estrous | 7000 | 2400 | 450 | 140 |
| 6-9 | Metestrous | 2000 | 910 | 1800 | ~40 |
| 6-16 | Anestrous | 2500 | 1100 | 140 | ~60 |

[a]Peak areas are roughly proportional to concentrations. Response factors were not known.
[b]Temporary instrument failure.

represented by peaks 46 and/or 47 may be related to the state of estrus, and may be important in communication between female and male dogs. Further work is needed to identify these constituents and determine their effects on male dogs, as well as to determine the degree of reproducibility of relative concentrations of all of the the observed constituents, with more animals and over a longer period of time.

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# ENANTIOMERIC COMPOSITION OF AN ALARM PHEROMONE COMPONENT OF THE ANTS *Crematogaster castanea* AND *C. liengmei*

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**Abstract**—The enantiomeric composition of 3-octanol produced by the ants, *Crematogaster castanea* Forel and *C. liengmei* F. Smith, as a component of their alarm pheromone complex, has been determined to be *S*-(+)-3-octanol by gas chromatography of the diastereomeric *S*-(+)-3-octanyl *R*-(+)-*trans*-chrysanthemates.

**Key Words**—Crematogaster castanea, C. liengmei, Hymenoptera, Formicidae, ant, alarm pheromone, enantiomers, 3-octanol.

## INTRODUCTION

A number of species of the myrmecine ant, *Crematogaster*, secrete an alarm pheromone from the mandibular glands that contains 3-octanone and 3-octanol as major components (Crewe et al., 1969, 1970). As biological activity is often dependent on the enantiomeric composition of a chiral compound, the ratio of enantiomers of the 3-octanol in two species of *Crematogaster* has been determined by gas chromatography of the 3-octanyl *R*-(+)-*trans*-chrysanthemates. The use of *R*-(+)-*trans*-chrysanthemoyl esters for the resolution of a number of synthetic chiral alcohols has been described (Brooks et al., 1972) and the method has been applied recently to the determination of the enantiomeric composition of 3-octanol from three species of *Myrmica* ants (Attygalle et al., 1983).

## METHODS AND MATERIALS

*Collection of Ants.* A nest of *Crematogaster castanea* F. Smith was cut from a branch of a tree in Nylsvlei, Transvaal, and a number of colonies of *C.*



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*liengmei* Forel were obtained from cracks in old fence poles at Margate, Natal. These colonies were maintained in the laboratory on a diet of water, honey, crickets, and grasshoppers.

trans-*Chrysanthemic Acid.* A natural pyrethrum extract was hydrolyzed with base, extracted three times with ether, acidified to pH 2 with HCl and reextracted three times with ether. These latter ether extracts were pooled and concentrated on a rotary evaporator. The yellow-brown oil was separated on a Florisil column by eluting first with *n*-hexane, followed by *n*-hexane–ether (9 : 1), and ether. Collected fractions were monitored by development on silica gel TLC chromatoplates in *n*-hexane–ether (1 : 1) and staining in $I_2$ vapor. The presence of *trans*-chrysanthemic acid at an $R_f$ of about 0.6 was checked against authentic *cis*-chrysanthemic acid. Rerunning the Florisil column on pooled fractions of the partially purified *trans*-chrysanthemic acid resulted in a pale yellow oil that gave one single spot on TLC with $I_2$ vapor. Comparison of proton and carbon-13 NMR spectra of this oil in $CDCl_3$ against spectra obtained for authentic *cis*-chrysanthemic acid confirmed its structure and purity.

*Preparation of Derivatives.* Worker ants of *C. liengmei* were steam distilled and continuously liquid–liquid extracted with *n*-pentane. Heads of worker ants of *C. castanea* were extracted directly with *n*-hexane. Solvent was removed carefully, under a stream of $N_2$, and the residue immediately treated with the acid chloride of the *trans*-chrysanthemic acid, obtained by reaction with oxalyl chloride. Authentic *R*-(−)-2-octanol, *S*-(+)-2-octanol, and racemic 3-octanol were derivatized in the same way.

*Gas Chromatography.* Three different capillary columns were used for each analysis: 12-m SE-30 (180°), 25-m BP-1 (J&W) (170°), and 25-m BP-20 (S.G.E.) (180°).

## RESULTS

*3-Octanone and 3-Octanol.* The solvent extracts of *C. castanea* and *C. liengmei* gave chromatograms on all three columns having two major peaks eluting at retention times corresponding to 3-octanone and 3-octanol. Treatment of a portion of the *C. liengmei* extract with $NaBH_4$ resulted in the loss of the peak corresponding to 3-octanone and an increase in the relative area of the peak corresponding to 3-octanol. These results, together with the published data of Crewe et al. (1969, 1970), confirm the presence of 3-octanone and 3-octanol in these species.

trans-*Chrysanthemic Acid.* NMR established the purity and structure of the *trans*-chrysanthemic acid. Reaction of the acid chloride of the *trans*-chrysanthemic acid with either *R*-(−)- or *S*-(+)-2-octanol gave gas chromatograms on each column with a single peak. Previously obtained mass spectral data on different columns had confirmed that these peaks were due to 2-octanyl *trans*-chrysanthemates. The retention times of these peaks on two of the columns were

as follows: *S*-(+)-2-octanyl *trans*-chrysanthemate: BP-1 (170°) 9.80 min, BP-20 (180°) 9.26 min; and *R*-(−)-2-octanyl *trans*-chrysanthemate: BP-1 (170°) 10.14 min, BP-20 (180°) 9.55 min. The appearance of these two resolved peaks in these chromatograms established the *trans*-chrysanthemic acid to be enantiomerically pure.

*3-Octanol and Ant Extracts.* Racemic 3-octanyl *R*-(+)-*trans*-chrysanthemate gave two peaks on the BP-1 column (170°) at 9.19 and 9.41 min and on the BP-20 column (180°) at 8.56 and 8.69 min. Derivatized extracts from both ant species gave only one peak corresponding to the earlier of the two 3-octanyl peaks. This peak was well resolved from any other peaks in the chromatogram. Coinjection of the racemic 3-octanyl *R*-(+)-*trans*-chrysanthemate and the derivatized ant extract resulted in an increased peak height for the first of the two peaks. Therefore, the chirality of the 3-octanol in both ant species corresponded to that of the 3-octanol in the earlier eluting diastereomer.

## DISCUSSION

Workers of both *C. castanea* and *C. liengmei* produce 3-octanone and 3-octanol as major components of their alarm pheromone complex. The gas chromatographic results reported here confirm the earlier work of Crewe et al. (1969, 1970) on *C. castanea* and add the species, *C. liengmei*, to the list of *Crematogaster* species producing these related compounds.

The *trans*-chrysanthemic acid purified from the pyrethrum extract was shown to be pure from its NMR spectrum. As it gave two distinct peaks on reaction with the enantiomers of 2-octanol, it was concluded to be the *R*-(+)-*trans* enantiomer known to occur in an esterified form in pyrethrin I. As (+)-2-octanol is the *S* enantiomer, and (−)-2-octanol is the *R* enantiomer (Brooks et al., 1972), the results obtained illustrate that the *R–S* diastereomer elutes before the *R–R* diastereomer as previously shown by Brooks et al. (1972). Furthermore, Attygalle et al. (1983) found that the *R–S* diastereomer of the 3-octanyl derivative elutes before the *R–R* diastereomer. Therefore, these two species of *Crematogaster* produce *S*-(+)-3-octanol exclusively. This finding is in contrast to the results of Attygalle et al. (1983) for three species of *Myrmica*, which produce a mixture containing mainly *R*-(−)-3-octanol. Even though species of both genera produce 3-octanol, there is nevertheless a high degree of specificity in their biosynthetic mechanism and, most likely, in their receptor mechanism. The enzymatic reduction of 3-octanone requires *Si*-face addition of a hydride ion to form *S*-3-octanol, and *Re*-face addition to form *R*-3-octanol. The pure enantiomers of 3-octanol are not available to the author at this time for any behavioral studies, but their enzymatic synthesis is being attempted.

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# CHEMICAL DETECTION OF SEX AND CONDITION IN THE CRAYFISH *Orconectes virilis*

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**Abstract**—Individual crayfish *(Orconectes virilis)* were tested for responses to water containing conspecific individuals of several sex-status categories. Isolated males did not react to "self" water but did show aggressive postures while isolated, nonself male water was introduced. Males' responses to female water was different from responses to male water. Water from aggressing males elicited fewer agonistic postures and more "neutral" postures. Females showed little difference in response to waters from different categories of conspecifics.

**Key Words**—Chemical detection, *Orconectes virilis*, crayfish, sex recognition, stress.

## INTRODUCTION

The utilization of chemical information by animals is widespread in all environments, and freshwater systems are no exception. Changes in behavior following detection of waterborne chemicals produced by conspecifics have been documented for a variety of species and behavioral situations. The responses of crayfish (Family Cambaridae) to waterborne substances illustrate well the ways in which chemicals can influence behavior.

Recognition of maternal condition by young crayfish was demonstrated by Little (1975, 1976) for *Orconectes sanborni* and *Procambarus clarkii.* Tierney and Dunham's (1982) study of *Orconectes virilis* and *O. propinquus* illustrates species recognition by chemical means, while Thorp and Ammerman (1978) provided evidence for the detection of a stress-induced chemical by conspecific individuals of *Procambarus acutus.* Recognition of sex by chemical means was reported for male *Procambarus clarkii* (Ameyaw-Akumfi and Hazlett, 1975), but Itagaki and Thorp (1981) concluded that such a capability by *P. clarkii* was not demonstrable by their own experiments.



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The following experiments were designed to examine a number of questions concerning responses to waterborne chemicals in the crayfish *Orconectes virilis*. Specifically, the responses of isolated males and females to water containing a member of either the same or opposite sex was examined as were possible changes in such responses after pair bonds were formed. In addition, the responses of males to "self" water and water in which agonistically interacting conspecific males were housed were recorded.

## METHODS AND MATERIALS

The experiments were carried out between October and mid-November, 1983, with specimens of *Orconectes virilis* which had been collected from a stream in Pinckney, Michigan (see Hazlett, et al., 1974, for some ecological details on this population). Specimens were held in unisexual communal aquaria prior to use and were tested within three weeks of collection. Pair bonds are normally formed at this time of year by these crayfish (Ameyaw-Akumfi, 1976; Hazlett, 1983). All individuals were sexually mature (45–60 mm cephalothorax length), and all males were form I.

Animals to be tested were placed in visually isolated 50 × 25-cm aquaria with 10 cm of well water. "Source" animals were placed in 18 × 26-cm aquaria with 15 cm of well water and a clay pot for shelter. Both test and source aquaria were continually aerated. Both test and source animals were allowed to settle in their aquaria for 24 hr prior to testing to avoid any stress associated with transfer. Tests were run between 1300 and 1700 hr, and the crayfish were fed daily after testing.

For each test, 5-mm-diameter tubing was arranged from a source aquarium to the test aquarium emptying in the corner opposite the test crayfish's initial position. The observer positioned himself about $1\frac{1}{2}$ m from the test tank. After a 10-minute period, a peristaltic pump (Masterflex Speed Controller) was activated which delivered water from the source to test aquarium at the rate of 250 ml/10 min. For the next 10 min, the posture of the test crayfish was recorded. The tubing was rinsed with well water between tests.

The postures were categorized into three levels for each of three parts of the crayfish. The chelipeds were either lowered (long axis of mani between parallel to substrate and a 20° angle with substrate, tips of dactyls in contact with substrate), neutral (long axis of mani at 25–40° angle to the substrate, tips within a few millimeter of substrate), or raised (long axis of mani parallel to substrate or higher with tips at least 10 mm from substrate). The chephalothorax was categorized as either lowered (coxae of walking legs in contact with substrate), neutral (normal walking position, coxae of walking legs less than 10 mm above substrate) or raised (body elevated, coxae of walking legs 10 mm or more

TABLE 1. CONDITION OF OBSERVED CRAYFISH AND SOURCE WATER AQUARIUM FOR DIFFERENT TEST SITUATIONS

| Observed Crayfish | Source water condition |
|---|---|
| 1. Isolated male | isolated male |
| 2. Isolated female | isolated male |
| 3. Isolated male | isolated female |
| 4. Isolated female | isolated female |
| 5. Isolated male | same male (self) |
| 6. Isolated male | two aggressing males |
| 7. Paired male | isolated male |
| 8. Paired female | isolated male |
| 9. Paired male | isolated female |
| 10. Paired female | isolated female |

above substrate). The abdomen was categorized as curled (telson under anterior segments of abdomen), neutral (abdomen almost straight but telson perpendicular to substrate), or extended (abdomen straight with telson parallel to substrate, uropods spread). The data for each test were the number of seconds, during the 10-min observation period, with the parts in each of the described positions. The results were analyzed by ANOVAs with pairwise comparisons. During the pretrial period, crayfish were usually in a lowered, chelae-down position in a corner of the aquarium.

Each test crayfish was observed under two to four test conditions but was used in any one type of test only once. The sequence of test conditions of isolated test animals was randomized, although the paired test conditions were run later for the most part to allow testing of individuals before and after pairing.

The status and conditions of test and source animals are listed in Table 1. There were 11 replicates for each of the 10 types of tests. For the "self" tests with male *O. virilis*, water from the test animal's aquarium was circulated by the peristaltic pump back to the same aquarium. For the "agonistic stress" source condition, a second male *O. virilis* was placed in the source aquarium with a previously isolated male just ten minutes before the start of observations; in all cases the animals in the source aquarium actively engaged in agonistic interactions (Rubenstein and Hazlett, 1975) for the duration of the observations. For the "paired" test animals, a male and female were placed together with a large clay pot for a shelter. They were allowed to interact for at least two days and were judged to be paired if they cohabited the pot with no aggressive displays exchanged for two days or if they were observed in copulation. The pair was observed together (in the same aquarium) during a test and postures recorded for both when water from a source aquarium was introduced.

## RESULTS

The means of the number of seconds spent in the various postures during the observation periods are shown in Table 2. The intermediate abdomen posture was observed so infrequently that it is not included in the table. The "neutral" postures of the chelipeds and body were also infrequent overall. Thus the crayfishes' parts were primarily raised or lowered. This means the two measures (time spent raised, time spent lowered) are not independent (Cane, 1963) since the value of one sets the value of the other in most situations. For this reason, only one set of variables can be examined and the raised/extended variables were analyzed by ANOVA (Table 3), since those postures were the most different from the resting postures observed during the pretrial periods. Only those comparisons which could answer a biologically interesting or meaningful question were examined (i.e., a number of comparisons which made no sense are not included in Table 3).

As shown in Table 3, there were a number of significant differences among the treatment groups in the times spent with the chelipeds raised (overall $F$ statistic $= 3.13$, $P = 0.0023$) and body raised (overall $F = 3.07$, $P = 0.0027$). The pattern of differences was very similar for these two postures. The times spent with the abdomen extended did not vary among the treatment groups (overall $F = 1.63$, $P = 0.1150$).

The responses of female *O. virilis* showed very few differences among treatments. Females showed no differences in responses to male and female water (2 vs. 4). Paired females did not differ from isolated females in their responses to female water (4 vs. 10). Paired females had the abdomen extended less than isolated females when male water was introduced ($P = 0.03$) but otherwise there were no differences.

Male *O. virilis* responded differently, although marginally, to male and female water in the time spent with the chelipeds raised and body raised (1 vs. 3). Males' responses to isolated males was different from that of females' (1 vs. 2) but there was no difference for isolated males and females in the responses to female water (3 vs. 4).

Males clearly distinguished between water inhabited by another male *O. virilis* and water inhabited by that same individual male (1 vs. 5). "Self" water introduction elicited no visible responses from test males whereas water from similar-but-nonself male *O. Virilis* frequently elicited raised chelipeds and cephalothorax.

Males also behaved very differently when water from aggressing males was introduced as compared to water from an isolated male (1 vs. 6). Raised chelipeds and body postures were less frequent and neutral postures more common.

Males which were paired with a female behaved very differently towards male water when compared to the behavior shown by isolated males to male water (1 vs. 7). The responses to female water by paired and isolated males was

TABLE 2. MEAN NUMBER OF SECONDS (STANDARD DEVIATION) DURING WHICH CHELIPEDS, CEPHALOTHORAX, AND ABDOMEN WERE IN DESCRIBED POSITIONS[a]

| Sex | | Postures observed | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Observed crayfish | Source water condition | Chelipeds Raised | Body Raised | Abdomen Extended | Chelipeds Lowered | Body Lowered | Abdomen Curled | Chelipeds Neutral | Body Neutral |
| 1. M | M | 258 (253) | 272 (219) | 180 (221) | 342 (253) | 310 (201) | 420 (222) | 0 | 18 ( 67) |
| 2. F | M | 114 (119) | 130 (149) | 149 (186) | 405 (186) | 350 (211) | 427 (194) | 80 ( 97) | 119 (140) |
| 3. M | F | 138 (238) | 137 (238) | 221 (229) | 459 (235) | 407 (208) | 467 (229) | 3 ( 9) | 56 (121) |
| 4. F | F | 77 (112) | 57 ( 86) | 86 (136) | 473 (190) | 453 (199) | 513 (136) | 50 (101) | 89 (145) |
| 5. M | M (self) | 0 | 0 | 0 | 600 | 600 | 600 | 0 | 0 |
| 6. M | 2M | 49 (130) | 87 (124) | 89 (171) | 462 (218) | 397 (256) | 510 (171) | 89 (149) | 115 (174) |
| 7. Mp[b] | M | 55 (128) | 89 (162) | 74 (139) | 488 (206) | 489 (203) | 516 (150) | 56 (142) | 21 ( 71) |
| 8. Fp | M | 31 ( 77) | 31 ( 77) | 21 ( 72) | 554 ( 98) | 526 (169) | 579 ( 72) | 14 ( 32) | 42 (103) |
| 9. Mp | F | 17 ( 47) | 44 ( 92) | 22 ( 64) | 524 (116) | 489 (144) | 564 ( 80) | 59 (108) | 66 (105) |
| 10. Fp | F | 60 (114) | 76 (127) | 71 (129) | 511 (123) | 487 (171) | 528 (129) | 29 ( 56) | 37 ( 87) |

[a]Eleven individual *Orconectes virilis* served as replicates for each test condition.
[b]The p in test conditions 7–10 indicates the individuals observed were paired with a crayfish of the opposite sex.

TABLE 3. PROBABILITY VALUES ASSOCIATED WITH *F* VALUES FROM PAIRWISE COMPARISONS IN ANOVA

| | Chelipeds raised | Body raised | Abdomen extended |
|---|---|---|---|
| Male responses | | | |
| 1 vs. 2 | 0.012 | 0.016 | 0.622 |
| 1 vs. 3 | 0.052 | 0.032 | 0.469 |
| 1 vs. 5 | 0.000 | 0.000 | 0.011 |
| 1 vs. 6 | 0.001 | 0.004 | 0.186 |
| 1 vs. 7 | 0.001 | 0.003 | 0.103 |
| 3 vs. 4 | 0.290 | 0.178 | 0.469 |
| 3 vs. 9 | 0.043 | 0.125 | 0.090 |
| Female responses | | | |
| 2 vs. 4 | 0.499 | 0.190 | 0.287 |
| 2 vs. 8 | 0.142 | 0.084 | 0.035 |
| 4 vs. 10 | 0.767 | 0.738 | 0.805 |

different only in the frequency of raised chelipeds, paired males being less likely to show that posture (3 vs. 9).

## DISCUSSION

The results of these experiments indicate that male *Orconectes virilis* react differently to water in which conspecifics of several categories of sex and status were held. The lack of differential responses to male and female water by female *O. virilis* may well represent an inability to detect any possible differences in chemicals by females. However, the possibility that both visual and chemical stimuli must be detected at the same time, as in the blue crab *Callinectes sapidus* (Teytaud, 1971), cannot be ruled out. Alternatively, the measures taken may not have been appropriate to detect any differences in behavioral responses. In paired interactions, females tend to be somewhat aggressive towards both males and females (until pair bonds are formed with a male and then aggression, at least towards that male, decreases). In these tests, the postures associated with the agonistic behavior were just as frequently shown by females when male or female water was introduced. Response of females to "self" and stressed conspecifics will be measured in future tests.

As reported earlier for *Procambarus clarkii* (Ameyaw-Akumfi and Hazlett, 1975), males of *O. virilis* exposed to water in which a male conspecific was held maintained postures associated with aggression while introduction of female water was associated with lowered postures. It is interesting to note that males often maintained raised chelipeds and cephalothorax for 10–15 min after the introduction of male water had stopped, indicating either a persistence of a

behavioral tendency induced in the male or persistence of a chemical for some minutes (or both). Interestingly, in both the present study of *O. virilis* and Itagaki and Thorp's (1981) study of *P. clarkii*, the cheliped raised posture (the only one in common for the two studies) was (1) significantly different among all treatments (overall *F* value significant) and (2) often different for males and females exposed to the same treatment. The differences in conclusions of the two studies concern the responses of males to male and female water; in their comparison of male cheliped-raised duration, Itagaki and Thorp (1981, Table 2) state that the *P* value for that comparison was "$>0.05$"—which is also true in the present study where $P = 0.052$, i.e., greater than 0.05. Given that it was one of just three parameters tested in this study, it seems reasonable to suggest that the present results show a differential in cheliped-raised duration as well as in body-raised duration.

When males were paired, they did not respond with raised postures to male water. This difference in behavior must be interpreted with caution since there was a shelter in the test animals' aquarium and, in addition, the two animals in the observation aquarium presumably affected each other's behavior in a complex manner during the test period. Responses of one member of a pair following removal of the other member would be difficult to interpret, and those tests were not attempted.

The lack of any response to "self" water was very marked. This differential in time of postures maintained was clear and serves as an interesting control for the testing procedure. Discrimination of self as compared to others of the same species and sex is not surprising and has been reported for other crustaceans (Caldwell, 1979, 1984). The differences in behavior of male *O. virilis* exposed to self water and male *P. clarkii* exposed to water with no crayfish in the container (condition C of Itagaki and Thorp, 1981) is striking, even though the test conditions are similar in many ways. All male *O. virilis* spent the entire observation period in a lowered position, not moving. The male *P. clarkii* (Itagaki and Thorp, 1981, Figure 1) spent a considerable portion of the observation period "climbing" and showing "gross body movement" and executed more "chelae waving" than under any other condition. The control level behaviors of the crayfish appear to have been very different in the two studies.

This apparent difference in behaviors shown under control conditions in the two studies could be a function of the species of crayfish studied. Alternatively, the primarily lentic *P. clarkii* may not have adapted well to the flow-through system utilized by Itagki and Thorp (1981). In the present study and previous work (Ameyaw-Akumfi and Hazlett, 1975), a nonflowing system was chosen since it more closely resembles conditions in the natural environment. In the Pinckney populations of *Orconectes virilis*, adult males and females form pair bonds in the fall (Ameyaw-Akumfi, 1976) and the pair (plus occasional additional females) are partially sealed off in special burrows (Hazlett, 1983). At the time of year when the experiments were done, adult *O. virilis* live in an almost static

system. If chemical communication occurs at that time, it would have to operate in a nonflowing environment. See Hazlett (1984) and Thorp (1984) for additional discussion of this point.

The difference in responses of male *O. virilis* to water containing one, apparently undisturbed male conspecific and two, aggressing conspecifics is striking. Rather than showing raised postures, the response shown by crayfish exposed to an identifiable source of potential danger, the test individuals spent more time in the other positions. With due caution concerning an excess of statistical tests, the times spent in the neutral postures by test males were greater when the source water contained aggressively interacting crayfish ($P = 0.046$ for cheliped neutral, $P = 0.037$ for cephalothorax neutral comparing test conditions 1 and 6 by ANOVA). The crayfish behaved as if they detected a low level of disturbance of an unspecified nature, rather than responding to a known source of distrubance (by raising the chelipeds). Clearly there is an important design flaw in these comparisons since both the number (one vs. two) of crayfish and the conditions (undisturbed vs. aggressing) vary. Therefore the reason for differences in responses is somewhat open to interpretation. Since two males in an aquarium frequently aggress, a more complicated delivery of source water will be necessary to compare water containing one and two undisturbed male conspecifics. Unlike the responses to isolated males, the responses to stressed males appeared to cease within a minute after the introduction of water was stopped, possibly indicating shorter persistence of the chemical(s).

In some pilot tests, before the peristaltic pump was utilized for water delivery, rapid changes in test animal behavior toward isolated male water was noticed when the source male was disturbed by experimenter manipulation of the tubing to the source aquarium. The raised postures which had been shown towards undisturbed male water ceased within seconds following disturbance of the source male. Tests to determine if other means of source animal disturbance result in a detection of chemicals by conspecific individuals are planned.

The responses of crayfish to water containing disturbed conspecifics is quite distinct from the classic Schreckstoff situation (von Frisch, 1938), where animals respond to physically damaged conspecifics (Atema and Stenzler, 1977; Landauer and Chapnick, 1981; Stenzler and Atema, 1977). Responses to undamaged but disturbed conspecifics have been reported for other crustaceans. Thorp and Ammerman, (1978) reported responses to stress pheromones in the crayfish *Procambarus acutus*. Hazlett (1966) demonstrated avoidance by the hermit crab *Clibanarius tricolor* of water from an aquarium containing stressed individuals of a fiddler crab (*Uca rapax*) or aggressing individuals of a spider crab (*Mithrax verrucosus*). Clearly, additional tests on crustaceans, including *Orconectes virilis* and other crayfish, are needed to differentiate between responses to generalized stress-related chemicals and alarm pheromones.

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# EFFECTS OF DITERPENE ESTERS OF TIGLIANE, DAPHNANE, INGENANE, AND LATHYRANE TYPES ON PINK BOLLWORM, *Pectinophora gossypiella* SAUNDERS (LEPIDOPTERA: GELECHIIDAE)

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**Abstract**—Twenty esters, representing the biogenetically related tigliane, daphnane, ingenane, and lathyrane series of diterpenes, were screened for growth-inhibitory and insecticidal effects on newly hatched larvae of the North American cotton pest, *Pectinophora gossypiella* (pink bollworm). Among the tigliane derivatives tested, only 12-*O*-tetradecanoylphorbol-13-acetate and 12-*O*-(2-methyl)butyrylphorbol-13-decanoate, of seven phorbol diesters isolated from croton oil by a new procedure involving droplet countercurrent chromatography, were active against *P. gossypiella* as both growth inhibitors and insecticides. The effects of the former compound were not significantly diminished by acetylation of its C-20 primary hydroxy group. Three other croton oil phorbol diester constituents, as well as daphnetoxin and daphnetoxin-5,20-diacetate, exhibited activity as growth inhibitors, but not as insecticidal agents, at the doses used. None of the ingenane or lathyrane derivatives investigated was active in either respect. 12-*O*-Tetradecanoylphorbol-13-acetate was found to cause 100% mortality on second-stadium larvae of *Culex pipiens* at 0.6 ppm, but exhibited less significant effects on *Oncopeltus fasciatus* (second-stadium nymphs) and *Tribolium confusum* (adults) when applied at higher doses.

**Key Words**—Growth-inhibitory agents, insecticides, pink bollworm, *Pectinophora gossypiella*, Gelechiidae, Lepidoptera, diterpene esters, croton oil, *Croton tiglium*, Euphorbiaceae, 12-*O*-tetradecanoylphorbol-13-acetate, 12-*O*-(2-methyl)butyrylphorbol-13-decanoate, daphnetoxin, ingenol esters, ingol esters, mortality, house mosquito, *Culex pipiens*.



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## INTRODUCTION

Plants, unable to anticipate all types of adverse predation, may in consequence be expected to evolve defensive secondary metabolites to combat as a diverse a range of herbivores as possible (Rhoades, 1979). Thus, a genus or family of plants committed to the production of related toxic substances can be expected to elicit deleterious effects on exposure to a variety of animal taxa. Plants representing several genera of the families Euphorbiaceae and Thymelaeaceae are well known to be skin irritants or be otherwise toxic to mammalian species (Kingsbury, 1964). Several species in these two families that are active in this regard have been of prime interest to biologists and chemists owing to their unusual biological activities and the unstable and complex nature of the active chemical constituents involved.

The best known example is that of croton oil, the oil expressed from the seed of *Croton tiglium* L. (Euphorbiaceae). Historical use of this oil involved its application as a potent purgative and counterirritant as well as a piscicide and arrow poison. Chemical studies to elucidate the nature of the toxic principle(s) of croton oil were potentiated in 1941, when the oil was found to be a potent tumor-promoting agent for mouse skin (Berenblum, 1941). Diesters of the tetracyclic polyfunctional diterpene alcohol, phorbol, were later shown to be the agents responsible for its irritant and tumor-promoting activity (Hecker and Schmidt, 1974). The most potent and abundant tumor-promoting constituent of croton oil, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is used extensively in current biochemical studies on carcinogenesis. The biological activities of TPA have recently been reviewed (Boutwell, 1974; Scribner and Süss, 1978; Diamond et al., 1980; Blumberg, 1980a,b; Slaga et al., 1981), and include increased protein, prostaglandin, and polyamine synthesis: alteration of membrane characteristics and cellular differentiation; effects on cell division; and induction of neoplastic transformation.

Recently, phorbol-ester specific receptors have been found in mammalian tissues (Delclos et al., 1980; Shoyab et al., 1981; Hergenhahn and Hecker, 1981). However, few diterpenes isolated from plants have been assayed for their biological activity in insects (Wagner et al., 1983). The insecticidal activities of the phorbol esters that possess a tigliane carbon skeleton and of the biogenetically related diterpene esters, based on the hydrocarbons daphnane, ingenane, and lathyrane, do not appear to have been studied, despite previous mention of the use of croton oil as an insecticide in India (Dastur, 1956) and China (Roark, 1947).

This paper describes the isolation and testing of seven phorbol diesters (compounds I–VII), obtained from croton oil using a new procedure involving droplet countercurrent chromatography; two phorbol triesters (compounds VIII and IX); two daphnane esters (compounds X and XI); three ingenol esters (compounds XII–XIV), and six ingol esters (compounds XV–XX) (Figures 1 and 2).

Tigliane diterpenes

| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| I | Tetradecanoate | Acetate | H |
| II | Dodecanoate | Acetate | H |
| III | Decanoate | Acetate | H |
| IV | α-Methylbutyrate | Decanoate | H |
| V | Acetate | Decanoate | H |
| VI | Tiglate | Isobutyrate | H |
| VII | Tiglate | Acetate | H |
| VIII | Tetradecanoate | Acetate | Acetate |
| IX | Acetate | Acetate | Acetate |

Daphnane diterpenes

| Compound | $R_1$ | $R_2$ |
|---|---|---|
| X | H | H |
| XI | Acetate | Acetate |

FIG. 1.

The primary organism used in this study was the pink bollworm, *Pectinophora gossypiella* (Gelechiidae) Saunders. Additional tests with one of the phorbol diesters, 12-*O*-tetradecanoylphorbol-13-acetate (I) were conducted against several other species of insects.

Ingenane diterpenes

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| XII | Deca-2,4,6-trienoate | H | O-Angelate | OH |
| XIII | Angelate | H | O-Acetate | H |
| XIV | Acetate | Acetate | H | O-Acetate |

Lathyrane derivatives

| Compound | $R_1$ | $R_2$ |
|---|---|---|
| XV | Acetate | Benzoate |
| XVI | Acetate | Tiglate |
| XVII | Benzoate | Methyl |
| XVIII | Tiglate | Methyl |
| XIX | Tiglate | H |
| XX | Acetate | Acetate |

FIG. 2.

## METHODS AND MATERIALS

### *Bioassays*

The various diterpene esters (I–XX) were assayed for growth inhibition and insecticidal activity with an artificial diet assay. The compounds were dissolved in glass-distilled acetone, applied to nonnutritive $\alpha$-cellulose, evaporated to dryness, and added to the components of a meridic artificial diet, including solid

nutrients (casein, sucrose, wheat germ, Wesson salts), vitamins (C and B complex), and agar (Chan et al., 1978). Newly hatched larvae (first stadium) of the lepidopterous agricultural pest *Pectinophora gossypiella* (pink bollworm) were placed singly on portions of the diet in two-dram scintillation vials and placed in a dark incubator at 28°C and 60% relative humidity. Daily observations were made and larval weights were determined after 12 days (equivalent in time to fourth-stadium control larvae). This procedure was followed to determine $LC_{100}$ values (the dose causing 100% death) and $EC_{50}$ values (the dose causing 50% inhibition of growth). Growth inhibition was determined as the percentage difference in larval wet weight between control and treated insects. Twenty insects were treated at each of at least four doses of individual compounds. $EC_{50}$ values were derived from log-dose-probit lines fitted by eye (Finney, 1952).

Additional bioassays were conducted with 12-*O*-tetradecanoylphorbol-13-acetate (I), including an immersion assay against the second-stadium larvae of the house mosquito, *Culex pipiens* L., and topical assays against second-stadium nymphs of the milkweed bug, *Oncopeltus fasciatus* Dallas, and adults of the confused flour beetle, *Tribolium confusum* Jacquelin duVal.

The immersion assay used was that previously described (Klocke et al., 1984). Compound I was dissolved in 0.1% glass-distilled acetone in distilled water at various concentrations. Second-stadium *Culex pipiens* were transferred (5 larvae/10 ml test solution) into 1-oz plastic cups using a 1 × 1-in. circle of ordinary window screen. Each treatment was replicated four times and the minimum concentration that caused 100% mortality ($LC_{100}$) within 72 hr at 28°C and 16:8 light–dark photoperiod was determined.

The topical assay with *Oncopeltus fasciatus* has also been previously described (Klocke et al., 1984). Second stadium nymphs were temporarily anesthetized with $CO_2$ and topically treated on the dorsum of the abdomen with 1 μl of a glass-distilled acetone solution of compound 1. The treated insects were transferred to rearing jars with sunflower seeds and water at 28°C, 60% relative humidity, and 16:8 light–dark photoperiod for the 10-day duration of the assay period (sufficient time for the control insects to undergo two molts). The assay was repeated three times with four treatments using 5–10 nymphs/treatment.

The topical assay with *Tribolium confusum* entailed anesthetizing adults (of all ages) with $CO_2$ and topically treating them on the dorsum of the abdomen with 1 μl of a glass-distilled acetone solution of compound I. The treated insects were transferred to rearing jars containing whole wheat flour at 28°C, 60% relative humidity, and 16:8 light–dark photoperiod for the 10-day duration of the assay period. The assay was repeated three times with four treatments using 10 adults per treatment.

### *Test Compounds*

Test compounds were either isolated from their natural source utilizing a combination of droplet countercurrent chromatography, low-pressure reversed-

phase chromatography and/or preparative thin-layer chromatography (compounds I–VII, XII, XIII, XV–XIX) or partially synthesized by acetylation of TPA (I) or certain diterpene ester hydrolysates (compounds VIII, IX, XIV, XX). In addition, daphnetoxin and daphnetoxin-5,20-diacetate (compounds X and XI, respectively) were donated. Each test compound was tested for purity in several TLC systems prior to bioassay. The individual test compounds were obtained as described below.

*Tigliane Derivatives*

*Compounds I–VII.* These phorbol diesters were isolated from croton oil purchased from Sigma Chemical Co., St. Louis, Missouri, in 100-g batches (Lot 60F-0471). Croton oil (100 g) was dissolved in 100 ml hexane and partitioned four times with 50-ml aliquots of methanol–water (20:3). The aqueous-methanolic layers were combined and evaporated to dryness in vacuo at or below 40°C to give a hydrophilic residue [3% (w/w) yield]. This residue was positive for phorbol diesters when examined by TLC using chloroform–ethyl acetate (2:3) (v/v), exhibiting zones with $R_f$ values of 0.30 and 0.40 that stained a rusty brown color when treated with 60% sulfuric acid in ethanol followed by heat (110°C) (Hecker and Schmidt, 1974). All chromatographic fractions were monitored by TLC as described above, and all preparative TLC was performed on 20 × 20-cm silica gel GHLF plates with 250-μm-thick layers (Analtech Inc., Newark, Delaware), using chloroform–ethyl acetate as developing solvent (2:3) (v/v), unless otherwise specified.

Droplet countercurrent chromatography (DCCC) was performed at room temperature on a model A DCC chromatograph (Tokyo Rikakikai, Tokyo, Japan), using a solvent system of hexane–diethyl ether–*n*-propanol–absolute ethanol–water (17:40:15:25:20) (v/v). The sample, 1.0 g of the croton oil hydrophilic residue, was introduced when dissolved in a mixture of the two solvent phases (1:1), using a 10-ml sample chamber, with the upper nonpolar layer acting as the mobile phase. The instrument was operated in the ascending manner. Fractions, 120 drops each, were collected in an automatic fraction collector, after the appearance of the mobile phase in the first DCCC glass panel. Selected fractions from the DCCC separation were combined and further fractionated by octylsilyl-bonded reversed-phase low-pressure liquid chromatography on a prepacked Lobar size A LiChroprep RP–8 column (E. Merck, Darmstadt, G.F.R.) using a stepwise gradient elution with a Milton Roy miniPump (Milton Roy Co., Riviera Beach, Florida.)

DCCC fractions 58–81 (combined dry weight 624 mg) were subjected to reversed-phase chromatography (RP–8a) using an initial solvent system of water–acetonitrile–methanol (1:1:2). The solvent was switched to 1:1:3 at fraction 80. RP–8a fractions 51–74 (35 mg) were chromatographed preparatively by TLC to afford 4 mg of compound III ($R_f$ 0.30). RP–8a fractions 75–104 (116 mg) were

similarly purified by preparative TLC to yield 19 mg of compound II ($R_f$ 0.30). Preparative TLC of RP–8a fractions 105–112 (48 mg) gave 15 mg of compound I and 12 mg of compound IV ($R_f$ 0.30 and 0.40, respectively). RP–8a fractions 113–185 (222 mg) were initially separated on two 2-mm-thick 20 × 20-cm silica gel 60 F-254 plates (E. Merck, Darmstadt, G.F.R.) which, after elution, required purification on three 250-$\mu$m TLC plates. From this second chromatographic step, 59 mg of compound I were isolated.

DCCC fractions 82–104 (82 mg) were separated by reversed-phase chromatography (RP–8b) using an initial solvent system of water–acetonitrile–methanol (3 : 3 : 4). This solvent system was changed to 1 : 1 : 2 at fraction 31, then to 1 : 1 : 3 at fraction 61. RP–8b fractions 81–113 (35 mg) were purified by preparative TLC to yield 10 mg of compound III and XVI mg of compound V ($R_f$ 0.30 and 0.40, respectively).

DCCC fractions 105–161 (137 mg) were purified using reversed-phase chromatography and preparative TLC as described previously (Marshall and Kinghorn, 1984), to afford compounds VI (12-*O*-tiglylphorbol-13-isobutyrate, 14 mg) and VII (12-*O*-tiglylphorbol-13-acetate, 24 mg).

The isolation of eleven phorbol diesters from croton oil and their chemistry has been reviewed (Hecker, 1971; Hecker and Schmidt, 1974). Compound I was the first of the diesters isolated and characterized (Hecker et al., 1964; Hecker and Bresch, 1965). Compounds II and III were subsequently described by Hecker and Schairer (1967). The structure elucidation of compounds IV and V was published in 1965 (Hecker and Kubinyi, 1965; Clarke and Hecker, 1965, respectively). Compound VI was not isolated from croton oil by the Hecker group (Hecker, 1971), while compound VII has recently been detected as a component of croton oil by HPLC-MS (Bauer et al., 1983), but was not fully characterized at that time. For ease of comparison, full physical and spectroscopic data are presented for all compounds I through VII in the following paragraphs.

*Compound I.* 12-*O*-Tetradecanoylphorbol-13-acetate; resin; $[\alpha]_D^{25}$ + 30° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 231 nm ($\epsilon$ 5883); IR, $\nu_{max}$ (AgCl, neat) 3425, 2924, 2854, 1723, 1717, 1699, 1376, 1262, and 1243 $cm^{-1}$; PMR (360 MHz, $CDCl_3$) $\delta$ 0.878 (3H, m, 18-$H_3$), 1.088 (1H, d, $J$ = 5.2 Hz, 14-H), 1.2–1.4 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.763 (3H, s, 19-$H_3$), 2.089 (3H, s, $OCOCH_3$), 2.455, 2.592 (2H, ABq, $J_{AB}$ = 18.9 Hz, 5-$H_2$), 3.240 (2H, m, 8-H, 10-H), 3.967, 4.062 (2H, ABq, $J_{AB}$ = 11.0 Hz, 20-$H_2$), 5.405 (1H, d, $J$ = 10.4 Hz, 12-H), 5.580 (1H, s, OH, $D_2O$ exchangeable), 5.690 (1H, d, $J$ = 5.6 Hz, 7-H) and 7.583 ppm (1H, s, 1-H); MS, $m/z$ 616 ($M^+$, 1%), 598 (2), 574 (4), 556 (4), 538 (5), 520 (2), 389 (13), 370 (8), 328 (99), 310 (100), 300 (12), 295 (11), 292 (24), 282 (16), 228 (33), 217 (87), 199 (42), 154 (36), 129 (31), 112 (40), and 83 (76).

*Compound II.* 12-*O*-Dodecanoylphorbol-13-acetate; resin; $[\alpha]_D^{25}$ +7° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 226 nm ($\epsilon$ 4925); IR, $\nu_{max}$ (AgCl, neat) 3395, 2924, 2860, 1729, 1380, 1260, 1178, 1110, and 1060 $cm^{-1}$; PMR (60 MHz, $CDCl_3$)

$\delta$ 0.88 (3H, m, 18-$H_3$), 1.1–1.6 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.73 (3H, bs, 19-$H_3$), 2.09 (3H, s, $OCOCH_3$), 2.33 (2H, bs, 5-$H_2$), 3.66 (2H, m, 8-H, 10-H), 4.11 (2H, bs, 20-$H_2$), 5.42 (1H, d, $J$ = 9.9 Hz, 12-H), 5.68 (1H, bd, $J$ = 4.7 Hz, 7-H) and 7.57 ppm (1H, bs, 1-H); MS, $m/z$ 588 ($M^+$, 0.5%), 570 (3), 546 (2), 528 (3), 510 (5), 389 (16), 370 (6), 328 (70), 310 (91), 292 (21), 282 (20), 228 (19), 217 (24), 199 (17), 154 (11), 125 (18), 112 (16), and 83 (100).

*Compound III.* 12-*O*-Decanoylphorbol-13-acetate; resin; $[\alpha]_D^{25}$ +61° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 232 nm ($\epsilon$ 5362); IR, $\nu_{max}$ (AgCl, neat) 3643, 2925, 2857, 1706, 1456, 1377, 1329, 1262, 1242, 1180, and 990 $cm^{-1}$; PMR (60 MHz, $CDCl_3$) $\delta$ 0.91 (3H, m, 18-$H_3$), 1.1–1.6 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.77 (3H, m, 19-$H_3$), 2.09 (3H, s, $OCOCH_3$), 2.52 (2H, bs, 5-$H_2$), 3.24 (2H, m, 8-H, 10-H), 4.01 (2H, bs, 20-$H_2$), 5.42 (1H, d, $J$ = 10.0 Hz, 12-H), 5.67 (1H, bd, $J$ = 4.7 Hz, 7-H) and 7.58 ppm (1H, bs, 1-H); MS, $m/z$ 560 ($M^+$, 1%), 542 (1), 518 (2), 500 (3), 482 (4), 464 (1), 389 (8), 370 (3), 328 (91), 310 (100), 292 (21), 282 (17), 228 (14), 217 (51), 199 (46), 154 (14), 125 (19), and 83 (82).

*Compound IV.* 12-*O*-($\alpha$-Methyl)butyrylphorbol-13-decanoate; resin; $[\alpha]_D^{25}$ +38° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 226 nm ($\epsilon$ 6547); IR, $\nu_{max}$ (AgCl, neat) 3400, 2926, 2854, 1738, 1722, 1713, 1695, 1460, 1378, 1330, 1269, 1235, 1185, 1155, 1075, and 1010 $cm^{-1}$; PMR (60 MHz, $CDCl_3$) $\delta$ 0.93 (3H, m, 18-$H_3$), 1.06 (1H, d, $J$ = 5.1 Hz, 14-H), 1.2–1.6 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.77 (3H, bs, 19-$H_3$), 2.53 (2H, bs, 5-$H_2$), 3.25 (2H, m, 8-H, 10-H), 4.01 (2H, bs, 20-$H_2$), 5.45 (1H, d, $J$ = 10.4 Hz, 12-H), 5.69 (1H, bd, $J$ = 5.2 Hz, 7-H) and 7.78 ppm (1H, bs, 1-H); MS, $m/z$ 602 ($M^+$, 2%), 584 (3), 501 (14), 482 (3), 464 (1), 430 (6), 412 (11), 398 (2), 328 (89), 310 (100), 292 (22), 282 (17), 228 (8), 217 (61), 199 (32), 155 (15), 112 (24), and 83 (54).

*Compound V.* 12-*O*-Acetylphorbol-13-decanoate; resin; $[\alpha]_D^{25}$ +48° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 227 nm ($\epsilon$ 8582); IR, $\nu_{max}$ (AgCl, neat) 3406, 2922, 2855, 1735, 1717, 1625, 1378, 1235, 1080, and 1020 $cm^{-}1$; PMR (60 MHz, $CDCl_3$) $\delta$ 0.88 (3H, m, 18-$H_3$), 1.06 (1H, d, $J$ = 5.3 Hz, 14-H), 1.2–1.5 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.75 (3H, bs, 19-$H_3$), 2.07 (3H, s, $OCOCH_3$), 2.55 (2H, bs, 5-$H_2$), 3.25 (2H, m, 8-H, 10-H), 4.01 (2H, s, 20-$H_2$), 5.34 (1H, d, $J$ = 10.1 Hz, 12-H), 5.67 (1H, bd, $J$ = 5.4 Hz, 7-H), 5.69 (1H, s, OH, exchangeable with $D_2O$) and 7.58 (1H, bs, 1-H); MS, $m/z$ 560 ($M^+$, 0.5%), 542 (0.5), 501 (1), 482 (0.5), 388 (1), 370 (2), 328 (15), 310 (20), 292 (5), 267 (5), 227 (6), 199 (8), 173 (12), 155 (12), 125 (11), and 83 (100).

*Compound VI.* 12-*O*-Tiglylphorbol-13-isobutyrate; resin; $[\alpha]_D^{25}$ +37° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 216 nm ($\epsilon$ 9469); IR, $\nu_{max}$ (AgCl, neat) 3418, 2979, 2925, 2878, 1706, 1457, 1390, 1377, 1335, 1260, 1160, 1135, and 1078 $cm^{-1}$; PMR (60 MHz, $CDCl_3$) $\delta$ 0.89 (3H, m, 18-$H_3$), 1.1–1.4 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.74 (3H, bs, 19-$H_3$), 1.84 (6H, m, tiglate methyls), 2.54 (2H, bs, 5-$H_2$), 3.27 (2H, m, 8-H, 1-H), 4.02 (2H, bs, 20-$H_2$), 5.55 (1H, d, $J$ = 10.3

Hz, 12-H), 5.70 (1H, bd, $J$ = 4.7 Hz, 7-H), 5.80 (1H, s, OH, exchangeable with $D_2O$), 6.83 (1H, m, tiglate methine) and 7.59 (1H, bs, 1-H); MS, *m/z* 516 ($M^+$, 2%), 498 (0.5), 474 (2), 473 (5), 428 (1), 417 (8), 410 (2), 398 (2), 392 (1), 328 (38), 310 (42), 292 (9), 282 (8), 217 (20), 199 (11), 173 (14), 125 (9), and 83 (100).

*Compound VII.* 12-*O*-Tiglylphorbol-13-acetate; resin; $[\alpha]_D^{25}$ +49° (c 0.09, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 220 nm ($\epsilon$ 10,529); IR, $\nu_{max}$ (AgCl, neat) 3425, 2922, 1722, 1712, 1695, 1378, 1325, 1260, 1130, and 1075 $cm^{-1}$; PMR (360 MHz, $CDCl_3$) $\delta$ 0.891 (3H, m, 18-$H_3$), 1.095 (1H, d, $J$ = 5.2 Hz, 14-H), 1.208 (3H, s, 16-$H_3$), 1.271 (3H, s, 17-$H_3$), 1.757 (3H, bs, 19-$H_3$), 1.797 (1H, d, $J$ = 7.0 Hz, tiglate methyl), 1.833 (1H, s, tiglate methyl), 2.099 (3H, s, $OCOCH_3$), 2.446, 2.610 (2H, ABq, $J_{AB}$ = 19.4 Hz, 5-$H_2$), 3.253 (2H, m, 8-H, 10-H), 3.963, 4.069 (2H, ABq, $J_{AB}$ = 12.9 Hz, 20-$H_2$), 5.457 (1H, d, $J$ = 10.3 Hz, 12-H), 5.628 (1H, s, OH, exchangeable with $D_2O$), 5.688 (1H, bd, $J$ = 4.5 Hz, 7-H), 6.841 (1H, m, tiglate methine) and 7.589 ppm (1H, s, 1-H); MS, *m/z* 488 ($M^+$, 2%), 470 (1), 446 (2), 410 (1), 392 (1), 389 (4), 370 (2), 328 (26), 310 (29), 292 (5), 282 (6), 228 (6), 227 (8), 217 (6), 187 (6), 173 (7), 139 (6), and 83 (100).

*Compound VIII.* 12-*O*-Tetradecanoylphorbol-13-acetate (I, 6 mg) isolated as described herein, was treated with pyridine–acetic anhydride (1:1, 1 ml) and stored overnight in an airtight vial at room temperature. Following removal of solvent under a stream of $N_2$, TLC revealed a single product, $R_f$ 0.59, when developed in $CHCl_3$–ethyl acetate (2:1), that was identified as the resinous 12-*O*-tetradecanoylphorbol-13,20-diacetate (VIII, 6.4 mg), on the basis of the following data: $[\alpha]_D^{25}$ +49° (c 0.1, $CHCl_3$); UV, $\lambda_{max}$ (EtOH) 231 nm ($\epsilon$ 9426); IR, $\nu_{max}$ (AgCl, neat) 3418, 2927, 1725, 1375, 1325, 1261, 1241, 1090, and 1025 $cm^{-1}$; PMR (60 MHz, $CDCl_3$) $\delta$ 0.91 (3H, m, 18-$H_3$), 1.07 (1H, d, $J$ = 5.0 Hz, 14-H), 1.26 (aliphatic side chain, 16-$H_3$, 17-$H_3$), 1.79 (3H, bs, 19-$H_3$), 2.05 (3H, s, $OCOCH_3$), 2.08 (3H, s, $OCOCH_3$), 2.48 (2H, m, 5-$H_2$), 3.26 (2H, m, 8-H, 10-H), 4.46 (2H, m, 20-$H_2$), 5.42 (1H, d, $J$ = 10.2 Hz, 12-H), 5.51 (1H, s, OH, exchangeable with $D_2O$), 5.71 (1H, bd, $J$ = 5.6 Hz, 7-H) and 7.60 ppm (1H, bs, 1-H); MS, *m/z* 658 ($M^+$, 2), 598 (8), 580 (3), 556 (10), 538 (26), 520 (42), 431 (77), 412 (5), 388 (16), 370 (100), 353 (10), 292 (5), 227 (6), 199 (5), 173 (10), 159 (12), 109 (17), and 83 (49).

*Compound IX.* Phorbol-12,13,20-triacetate (IX) was obtained on the acetylation of phorbol isolated from croton oil following hydrolysis, droplet countercurrent chromatography, and low-pressure reversed-phase chromatography (Marshall and Kinghorn, 1981).

*Daphnane Derivatives*

The daphnane derivatives, daphnetoxin (X), an active constituent of *Daphne mezereum* L. (Thymelaeaceae) (Stout et al., 1970), and the semisynthetic daphnetoxin-5,20-diacetate (XI), were kindly donated by Professor G. H. Stout.

### *Ingenane Derivatives*

Two ingenane derivatives, 3-*O*-*n*-(deca-2,4,6-trienoyl)-16-hydroxyingenol-16-angelate (XII) and 3-*O*-angelyl-16-hydroxy-20-deoxyingenol-16-acetate (XIII), were isolated from the latex of *Euphorbia hermentiana* Lem. (Euphorbiaceae) (Lin et al., 1983) by droplet countercurrent chromatography and preparative TLC. Compound XIV, ingenol-3,5,20-triacetate, was obtained from *E. hermentiana* as a semisynthetic derivative (Hickey et al., 1981).

### *Lathyrane Derivatives*

Five ingol esters, 8-*O*-benzoylingol-3,7,12-triacetate (XV), 8-*O*-tiglylingol-3,7,12-triacetate (XVI), 7-*O*-benzoyl-8-methoxyingol-3,12-diacetate (XVII), 7-*O*-tiglyl-8-methoxyingol-3,12-diacetate (XVIII) and 7-*O*-tiglylingol-3,12-diacetate (XIX) were isolated from *Euphorbia hermentiana* latex by droplet countercurrent chromatography and preparative TLC (Lin and Kinghorn, 1983). Compound XX, ingol-3,7,8,12-tetraacetate, was obtained by hydrolysis and subsequent acetylation and work-up of *E. hermentiana* latex (Hickey et al., 1981).

## RESULTS

The growth-inhibitory and insecticidal efficacies against *Pectinophora gossypiella* of 20 biogenetically related polyfunctional diterpene esters are shown in Table 1. These comprised tigliane (compounds I–IX), daphnane (compounds X and XI), ingenane (compounds XII–XIV), and lathyrane (compounds XV–XX) derivatives. The most active of the compounds tested was the tigliane diterpene, 12-*O*-tetradecanoylphorbol-13-acetate (compound I), which had a dietary $EC_{50}$ (effective concentration for 50% growth inhibition) of 3 ppm and a $LC_{100}$ (lethal concentration for 100% death) of 20 ppm. The acetylated derivative of compound I, 12-*O*-tetradecanoylphorbol-13,20-diacetate (compound VIII) was very similar to compound I in its activity, with $EC_{50}$ and $LC_{100}$ values of 4 ppm and 20 ppm, respectively. Compound IV, 12-*O*-($\alpha$-methyl)butyrylphorbol-13-decanoate, with an $EC_{50}$ of 7 ppm and a $LC_{100}$ of 40 ppm, was about twofold less active as a growth inhibitor and an insecticide than compound I. No $LC_{100}$ values were determined for compounds II (12-*O*-dodecanoylphorbol-13-acetate), III (12-*O*-decanoylphorbol-13-acetate), and V (12-*O*-acetylphorbol-13-decanoate) since only between 20% and 80% mortality occurred at 100 ppm, the highest dietary dose of these compounds tested. However, as a growth inhibitor, compound V proved to be about twofold more active ($EC_{50}$ of 26 ppm) than either compound II ($EC_{50}$ of 60 ppm) or compound III ($EC_{50}$ of 55 ppm), but almost fivefold less active than compound I. The other tigliane esters tested, including compounds VI (12-*O*-tiglylphorbol-13-isobutyrate), VII (12-*O*-tiglylphorbol-13-acetate), and

TABLE 1. GROWTH INHIBITORY AND INSECTICIDAL ACTIVITIES OF FOUR GROUPS OF POLYFUNCTIONAL DITERPENE ESTERS AGAINST NEWLY HATCHED LARVAE OF *Pectinophora gossypiella*

| Compound | $ED_{50}$(ppm)[a] | $LD_{100}$(ppm)[b] |
|---|---|---|
| I. Tigliane diterpenes | | |
| 12-*O*-Tetradecanoylphorbol-13-acetate (I) | 3 | 20 |
| 12-*O*-Tetradecanoylphorbol-13,20-diacetate (VIII) | 4 | 20 |
| 12-*O*-(α-Methyl)butyrylphorbol-13-decanoate (IV) | 7 | 40 |
| 12-*O*-Acetylphorbol-13-decanoate (V) | 26 | *c* |
| 12-*O*-Decanoylphorbol-13-acetate (III) | 55 | *c* |
| 12-*O*-Dodecanoylphorbol-13-acetate (II) | 60 | *c* |
| 12-*O*-Tiglylphorbol-13-isobutyrate (VI) | *d* | *d* |
| 12-*O*-Tiglylphorbol-13-acetate (VII) | *d* | *d* |
| Phorbol-12,13,20-triacetate (IX) | *d* | *d* |
| II. Daphnane diterpenes | | |
| Daphnetoxin (X) | 5 | *e* |
| Daphnetoxin-5,20-diacetate (XI) | 17 | *c* |
| III. Ingenane diterpenes | | |
| 3-*O*-*n*-(Deca-2,4,6-trienoyl)-16-hydroxyingenol-16-angelate (XII) | *d* | *d* |
| 3-*O*-Angelyl-16-hydroxy-20-deoxyingenol-16-acetate (XIII) | *d* | *d* |
| Ingenol-3,5,20-triacetate (XIV) | *d* | *d* |
| IV. Lathyrane diterpenes | | |
| 8-*O*-Benzoylingol-3,7,12-triacetate (XV) | *d* | *d* |
| 8-*O*-Tiglylingol-3,7,12-triacetate (XVI) | *d* | *d* |
| 7-*O*-Benzoyl-8-methoxyingol-3,12-diacetate (XVII) | *d* | *d* |
| 7-*O*-Tiglyl-8-methoxyingol-3,12-diacetate (XVIII) | *d* | *d* |
| 7-*O*-Tiglylingol-3,12-diacetate (XIX) | *d* | *d* |
| Ingol-3,7,8,12-tetraacetate (XX) | *d* | *d* |

[a] $EC_{50}$ values are the effective doses for 50% growth inhibition.
[b] $LC_{100}$ values are the lethal doses for 100% death and are expressive of comparative insecticidal activity of the test compounds.
[c] Between 20% and 80% death occurred at 100 ppm, the highest concentration tested.
[d] No effect was observed at 50 ppm, the highest concentration tested.
[e] Between 20% and 80% death occurred at 50 ppm, the highest concentration tested.

IX (phorbol-12,13,20-triacetate), showed no activity against the pink bollworm at dietary incorporation levels of up to 50 ppm.

Of the two daphnane derivatives tested, daphnetoxin (compound X) ($EC_{50}$ of 5 ppm) was about 3.5-fold more active than daphnetoxin-5,20-diacetate (compound XI) ($EC_{50}$ of 17 ppm). $LC_{100}$ values were not determined for either compound X or XI since only between 20% and 80% mortality occurred at the highest doses tested, which were 50 ppm for compound X and 100 ppm for

TABLE 2. RESPONSE OF THREE SPECIES OF INSECTS TO TREATMENT WITH 12-*O*-TETRADECANOYLPHORBOL-13-ACETATE (I)

| Insect species | Developmental stage | Dose | Response |
|---|---|---|---|
| *Culex pipiens* | Second-stadium larva | 0.6 ppm | $LC_{100}$[a] |
| *Oncopeltus fasciatus* | Second-stadium nymph | 32 μg | [b] |
| *Tribolium confusum* | Adult | 25 μg | NE[c] |

[a] $LC_{100}$ is the lethal dose for 100% death.
[b] Dose applications as high as 32 μg resulted in less than 80% mortality, which occurred either before or during molting. Survivors gained weight and developed more slowly than did the controls.
[c] NE signifies that no effect was observed.

compound XI. The potency of daphnetoxin (compound X) as a growth inhibitor for *Pectinophora gossypiella* was determined as being almost as great as that of compound I (Table 1). The ingenol esters, inclusive of compounds XII, [3-*O*-*n*-(deca-2,4,6-trienoyl)-16-hydroxyingenol-16-angelate], XIII (3-*O*-angelyl-16-hydroxy-20-deoxyingenol-16-acetate), and XIV (ingenol-3,5,20-triacetate), and the lathyrane derivatives, inclusive of compounds XV (8-*O*-benzoylingol-3,7,12-triacetate), XVI (8-*O*-tiglylingol-3,7,12-triacetate), XVII (7-*O*-benzoyl-8-methoxyingol-3,12-diacetate), XVIII (7-*O*-tiglyl-8-methoxyingol-3,12-diacetate), XIX (7-*O*-tiglylingol-3,12-diacetate), and XX (ingol-3,7,8,12-tetraacetate), showed no activity against the pink bollworm at dietary incorporation levels of up to 50 ppm.

The results of testing compound I against three additional species of insects are shown in Table 2. In an immersion assay with the house mosquito, *Culex pipiens*, the minimum dose causing 100% mortality ($LC_{100}$) to the second-stadium larvae was 0.6 ppm. In topical assays with the second-stadium nymph of the milkweed bug, *Oncopeltus fasciatus*, doses as high as 32 μg/insect caused less than 80% mortality, which occurred either before or during molting. Survivors gained weight and developed more slowly than did the controls. In topical assays with adults of the confused flour beetle, *Tribolium confusum*, doses as high as 25 μg/insect failed to result in an observable effect.

## DISCUSSION

The euphorbiaceous and thymelaeaceous diterpenes of the tigliane, daphnane, and ingenane classes have been hypothesized as being irritant defense substances (Schildknecht, 1981). These compounds, reported in all plant parts, are often biosynthesized in substantial amounts. Thus, phorbol esters, present in the seed oil of *Croton tiglium*, occur up to levels of 5.6% (w/w) (Hecker and Schmidt, 1974). Not only are these diesters toxic to mammalian systems, but we have

demonstrated them as being toxic to a lepidopterous species, *Pectinophora gossypiella.* Toxicological studies of these compounds in whole higher animals have been represented, for example, by measurement of mouse ear skin irritancy (Hecker et al., 1966), measurement of irritancy on human skin in open and closed patch testing (Hickey et al., 1981), and estimation of tumor-promoting activity in mice as tumor rates and average tumor yields (Hecker, 1963). Phorbol-12,13-diesters exhibit optimum irritant and tumor-promoting effects on mouse skin, if one of the ester groups contains a medium-chain-length (10–16 carbon) aliphatic carboxylic acid (Hecker, 1978).

In the present study, the nature of the ester substituent at both of positions C-12 and C-13 was found to influence the growth inhibitory and insecticidal activities of the phorbol diesters against *P. gossypiella* (Table 1). For example, while compounds I, II, and III all have an acetate at C-13, they differ in potency due to the chainlength of the ester substituent at C-12. Compound I, with a tetradecanoate moiety at C-12, was about 20-fold more active as a growth inhibitor than either compound II or compound III, which possess a dodecanoate and a decanoate, respectively, at C-12. Likewise, both compounds IV and V have a decanoate at C-13, but IV, which has a 2-methylbutyrate substituent affixed at C-12, was about fourfold more active as a growth inhibitor than V, which has an acetate at C-12. Conversely, compound V was about twofold more active than its positional isomer, compound III, with a decanoate at C-12 and an acetate at C-13. Compounds VI, VII, and IX, all having ester chain lengths of five carbons or less, were inactive against *P. gossypiella* at the dietary level of 50 ppm.

Acetylation of the 20 position of compound I to yield compound VIII had little effect on the potency against *P. gossypiella* (Table 1). This fact may be conjectured as being due to a possible in vivo partial hydrolysis of the ester functionality at the C-20 position, to produce the potent 12-*O*-tetradecanoylphorbol-13-acetate (compound I). It is known that esterification of the C-20 primary hydroxy group of phorbol-12,13-diesters results in the almost complete abrogation of their activities as mouse skin irritants and tumor-promoting agents (Hecker and Schmidt, 1974). It is interesting to observe, however, that acetylation of both the C-20 and the C-5 positions of compound X to yield compound XI resulted in substantially decreased growth inhibitory activity against *P. gossypiella* (Table 1). A similar effect was observed on the effects of these compounds on human skin. In closed patch tests, compound X produced severe inflammation while its 5,20-diacetate (compound XI) was devoid of these effects when applied at ten times the dose (Hickey et al., 1981).

Certain ingenane derivatives that are esters of the diterpene alcohols 16-hydroxyingenol and ingenol, are known to exhibit irritant and tumor-promoting activity for mouse skin (Adolf and Hecker, 1971, 1975). Although known to be a potent skin irritant and a weak tumor-promoting agent (Opferkuch and Hecker, 1982), compound XII was totally inactive in this assay when incorporated up to 50 ppm in the artificial diet used. However, it is possible that this labile compound

decomposed due to exposure to the atmosphere during the 12-day period of the bioassay employed. Since skin-irritant ingenane derivatives are far more widespread than tigliane and daphnane esters in the large genus *Euphorbia* (Kinghorn and Evans, 1975; Evans and Kinghorn, 1977), perhaps XII would be active if tested against other insect species. The ingol esters (XV–XX), based on the lathyrane skeleton, were also inactive against this insect larva at dietary levels of up to 50 ppm. While generally regarded as biologically inactive compounds as far as mouse skin irritancy and tumor promotion is concerned (Hickey et al., 1981), there is one report that describes weak cytotoxic activity by derivatives of 6,20-epoxylathyrol against cloned Morris rat hepatoma cells (Schroeder et al., 1979).

Since compound I was found to be the most potent of the compounds tested against *Pectinophora gossypiella,* it was further tested against other species of insects (Table 2). Against larvae of *Culex pipiens,* doses of 0.6 ppm or more caused 100% mortality. Thus, the mosquito larvicidal activity of compound I is about two- to fourfold more potent than that of some other biologically active natural plant constituents, including chromenes and isobutylamides (Klocke et al., 1984; Kubo et al., 1984). In topical assays with the nymphs of *Oncopeltus fasciatus* and adults of *Tribolium confusum,* compound I showed weak to no activity, respectively. However, one interesting observation was that compound I sometimes inhibited the ecdysis of treated *O. fasciatus* since the exuvial skin remained attached to the dead nymphs. It may be noted that this observation of lack of activity against *T confusum* is consistent with a recent report of the inactivity of *Croton tiglium* seed oil against adults of another member of this genus, *T castaneum* Herbst. (red flour beetle) (Khan et al., 1983).

The present study does not assay for any one particular type of toxicity or imply any mechanisms of action, but measures a general antibiosis. The toxicity data shown here for the pink bollworm, *Pectinophora gossypiella,* provides parallels and differences with biological effects known for the test compounds used in mammalian systems. These data may also be used to support the conjecture that these diterpene derivatives, biosynthesized by many species of the Euphorbiaceae and Thymelaeaceae, may act as one component in the defensive array of these plants against both mammalian and insect predators.

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# DETERMINING PHEROMONE CONTENT OF HAIRPENCILS FROM INDIVIDUAL VIRGIN MALES OF *Pseudaletia unipuncta* (HAW.) (LEPIDOPTERA: NOCTUIDAE)[1]

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**Abstract**—Levels of benzaldehyde recovered from virgin *Pseudaletia unipuncta* (Haw.) males were not influenced by (1) the time hairpencils remained in the solvent (1–72 hr), (2) anesthetization or agitation of males prior to excision of hairpencils, or (3) the time (photophase or scotophase) that hairpencils were excised. Thus the interindividual variability observed is not a methodological artifact. Most males had similar concentrations in both hairpencils, although in some cases only one hairpencil contained pheromone. In one case, a male with partially extruded hairpencils had no benzaldehyde at all. Neither pupal weight nor hairpencil length proved to be reliable indicators of pheromone content.

**Key Words**—Armyworm, benzaldehyde, benzyl alcohol, hairpencils, male response, Lepidoptera, Noctuidae, pheromone, *Pseudaletia unipuncta*, scent brushes.

## INTRODUCTION

Males of many noctuid species possess hairpencils (scent brushes) on the ventral surface of the abdomen (Weatherston and Percy, 1977), which are everted from abdominal pockets as the male approaches the female during courtship (Birch, 1974; Farine, 1982). Once everted, hairpencils release a pheromone which may enhance female receptivity (Birch, 1974) and/or act as a male-to-male inhibitor (Hirai et al., 1978; Hirai, 1980).

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Benzaldehyde has been identified as a component of volatile hairpencil secretions in 12 species from six genera of Noctuidae (Birch, 1974, and references therein; Weatherston and Percy, 1977), and Grant et al. (1972) reported it as the major component of the hairpencil secretion of *Pseudaletia unipuncta* (Haw.). Lesser amounts of benzyl alcohol and benzoic acid were also detected, but the acid was considered to be an oxidative degradation product (Grant et al., 1972). Work by Hirai (1980) confirmed that the only two components in *P. unipuncta* hairpencil secretions are benzaldehyde and benzyl alcohol. The latter is thought to be the precursor of the benzaldehyde (Clearwater, 1975; Hirai, 1980). Before males attain maturity at 25°C, two to three days after emergence, their hairpencils contain more benzyl alcohol than benzaldehyde (Grant et al., 1972); thereafter, benzaldehyde predominates (Hirai, 1980). Peak levels of benzaldehyde at four to seven days after emergence (Hirai, 1980) coincide with maximal behavioral response of males to the female pheromone at 25°C (Turgeon et al., 1983). Seabrook et al. (1979) also showed that electroantennogram sensitivity of male *P. unipuncta* antennae to benzaldehyde is greatest at this time.

The effects of abiotic and biotic factors on calling behavior of virgin females (McNeil and Turgeon, 1982; Turgeon, 1982; Turgeon and McNeil, 1982) constitute one aspect in an ongoing study of the reproductive biology of *P. unipuncta.* Research pertaining to males not only addresses the question of male responsiveness (Turgeon et al., 1983) but also the production of male hairpencil secretions under different experimental conditions. Several techniques for excising and extracting hairpencils have been reported (Grant et al., 1972; Hirai, 1980; Toth, 1982); however, in all cases the extracts analyzed combined hairpencils from several specimens and never of individual males. We carried out preliminary analyses of hairpencils from single males and observed considerable between-individual variability in the levels of pheromone detected. In order to establish a standard technique for the analysis of individual males in all future experiments, it was essential to determine if the observed variability reflected biological differences between individuals or resulted from the excision and extraction techniques employed. Experiments were therefore undertaken to clarify this question and to examine the relationship between the amount of benzaldehyde, pupal weight, and hairpencil length.

## METHODS AND MATERIALS

*P. unipuncta* males used in this study were reared on a modified Shorey and Hale (1965) pinto-bean diet at 25 ± 1.0°C, 65 ± 5% relative humidity, and a 16 : 8-hr light–dark cycle. Insects were sexed as pupae and each sex maintained separately thereafter. Pupae were weighed three days after pupation and, upon emergence, males were kept individually in 16-dram clear plastic vials where they had continual access to a cotton wick soaked in 8% sucrose solution. Only

4-day-old virgin males were used in this study, as our preliminary data showed that the amount of benzaldehyde/insect was greatest at this age.

*General Hairpencil Removal.* Males were decapitated and their hairpencils excised with fine forceps as carefully and quickly as possible. Hairpencils were transferred to a Teflon-capped 1-dram amber vial containing approximately 1 ml of methylene chloride. Vials were chilled in ice during the excision procedure and subsequently stored at $4 \pm 1°C$. Care was taken not to squeeze the abdomen or otherwise evert the hairpencils before excision. Hairpencils were removed from the solvent, dried, and their length recorded when all analyses had been terminated.

*Analyses.* To quantify benzaldehyde and benzyl alcohol, 50 $\mu$g of naphthalene in an aliquot of methylene chloride was added as an internal standard to each sample, after which the hairpencils were removed from the vial. A Hewlett-Packard 5710A gas chromatograph, fitted with flame ionization detectors and coupled to a Hewlett-Packard 3380A integrator, was used for analyses. The 6-ft $\times$ $\frac{1}{8}$-in. ID stainless-steel column, packed with 10% DEGS-PS on Supelcoport, (80/100 mesh) was operated isothermally at 170°C with a carrier gas (nitrogen) flow rate of 21.6 ml/min. Under these conditions, benzaldehyde has a retention time of 1.28 min, benzyl alcohol, 3.13 min, and naphthalene, 2.12 min. At least three injections of each sample were analyzed, and results averaged to yield amounts of benzaldehyde and benzyl alcohol per insect.

Benzaldehyde and benzyl alcohol were obtained from Aldrich Chemical Company (Canada) Ltd., Montreal, and methylene chloride, distilled in glass, from Burdick and Jackson Laboratories, Inc. Muskegon, Michigan.

*Treatments.* To determine the optimum extraction time (i.e., the amount of time needed for maximal extraction of hairpencil components by the solvent), the hairpencils of five males were excised, placed in individual vials containing solvent (both hairpencils from one insect per vial) and each of the five extracts sampled at 1, 2, 4, 12, 24, 48, and 72 hr after preparation.

As previously stated, we always decapitated males before hairpencil excision, but they often became very agitated during manipulations. We therefore tested four preexcision treatments: (1) anesthetization with $CO_2$, until immobile ($\simeq 2$ min), (2) immobilization by chilling at $-10°C$ for 15 min; (3) deliberate agitation (shaking the vial containing the insect for 10 sec); and (4) no deliberate immobilization or agitation. Ten males were used per treatment.

We also tested the effect of time of day on the amount of benzaldehyde found in hairpencil extracts, by excising hairpencils from 10 males at three different times: 1 and 7 hr after the onset of the scotophase and 5 hr after the onset of the photophase.

Furthermore, we examined the variation between the two hairpencils from the same individual, as well as the relationships between pupal weight, hairpencil length, and the amount of benzaldehyde for individual insects.

## RESULTS AND DISCUSSION

Benzyl alcohol was not recovered from any of the extracts, an expected result as hairpencils of 4-day-old males rarely contain detectable amounts of this compound (Grant et al., 1972; Hirai, 1980). Benzaldehyde was present in all but one individual (see below).

The data presented in Table 1 indicate that 1 hr is sufficient extraction time, as the amount of benzaldehyde/insect does not vary substantially over the 72-hr period. Consequently all extracts in subsequent experiments were analyzed within 72 hr after excision. Analyses of solutions containing known quantities of both benzyl alcohol and benzaldehyde, analyzed at intervals over a two-week period, showed no loss due to evaporation.

None of the preexcision treatments significantly affected the amount of benzaldehyde extracted/insect (Table 2). Insects in all other experiments were immobilized by chilling before decapitation and excision of hairpencils.

The amount of benzaldehyde/insect was likewise unaffected by the time, during either scotophase or photophase, that hairpencils were excised, as no significant differences between mean amounts of benzaldehyde from hairpencils excised 1 hr after scotophase onset, 7 hr after scotophase onset, and 5 hr after photophase onset were observed (Table 3). Turgeon et al. (1983) showed that the level of male responsiveness to low concentrations (10 and 30 $\mu$g) of a synthetic source of female pheromone is periodic, being greater during the scotophase (peaking 1 hr after onset) and lower during the photophase. Hairpencil benzaldehyde levels in isolated 4-day-old virgin males do not follow this pattern. However, changes in benzaldehyde levels have been reported with male age (Grant et al., 1972; Hirai, 1980) and with exposure to calling females (Hirai, 1980).

The hairpencils of one individual were partially exposed before dissection and contained no detectable benzaldehyde. Based on information in the literature (e.g., Birch, 1970b; Grant et al., 1972) and his own research, Clearwater (1975) proposed a pathway for the production of benzaldehyde in three noctuid species. Glycoside precursors are produced in the paired Stobbe glands which, early in adult life, empty their contents into the pockets containing the hairpencils, via gland ducts that open onto the proximal part of the hairpencils. The Stobbe glands become inactive and atrophy after discharging their contents into the pockets soon after adult emergence (Birch, 1970b; Grant et al., 1972), although there is sufficient pheromone in the pocket to recharge hairpencils several times following extrusion and subsequent retraction (Birch, 1970a). Once in the pocket, the glycoside is enzymatically degraded to produce the male pheromone, which may involve one or several steps (Clearwater, 1975). Clearwater (1975) proposed the cap cells at the base of the hairpencils as a potential site where the enzyme could be produced. However, Birch (1970b) reported that the scales lining the pockets of *Phlogophora meticulosa* (L.) appeared to be actively secreting and

TABLE 1. AMOUNT OF BENZALDEHYDE/INSECT EXTRACTED FROM HAIRPENCILS OF 4-DAY-OLD VIRGIN *Pseudaletia unipuncta* MALES[a]

| Benzaldehyde ($\mu g \pm SD$)/insect after specified period (hr)[b] | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 4 | 12 | 24 | 48 | 72 | $\overline{X}$ |
| 37.7 (±0.2) | 38.0 (±0.2) | 38.4 (±0.1) | 34.9 (±0.9) | 37.0 (±0.3) | 36.9 (±0.3) | 37.0 (±1.2) | 37.1 (±1.1) |
| 31.2 (±0.2) | 31.5 (±0.1) | 31.9 (±0.3) | 32.0 (±0.2) | 31.8 (±0.1) | 32.5 (±0.1) | 32.7 (±0.4) | 31.9 (±0.5) |
| 23.9 (±0.4) | 23.6 (±0.2) | 24.2 (±0.4) | 23.9 (±0.3) | 24.2 (±0.1) | 24.7 (±0.1) | 24.7 (±0.1) | 24.2 (±0.4) |
| 29.0 (±0.2) | 29.5 (±0.3) | 29.4 (±0.1) | 29.9 (±0.5) | 29.8 (±0.2) | 29.9 (±0.1) | 30.1 (±0.3) | 29.6 (±0.4) |
| 39.6 (±0.4) | 40.1 (±0.4) | 39.9 (±0.4) | 40.1 (±0.3) | 40.1 (±0.4) | 40.2 (±0.2) | 40.1 (±0.7) | 40.0 (±0.2) |

[a] Extracts from the same five males were sequentially sampled over 72 hr.
[b] Each insect weighed between 363 and 400 mg. Extraction solvent was methylene chloride.

TABLE 2. AMOUNT OF BENZALDEHYDE/INSECT FROM HAIRPENCILS OF 4-DAY-OLD VIRGIN *Pseudaletia unipuncta* MALES AFTER PREEXCISION TREATMENTS

| Treatments | Number of insects[a] | $\bar{X}$ benzaldehyde/insect[b] ($\mu g \pm SD$) |
|---|---|---|
| $CO_2$ anesthetization | 10 | 43.3 (±18.9) |
| Chilling | 10 | 40.2 (±16.0) |
| Agitation | 10 | 44.5 (±13.4) |
| No agitation | 10 | 39.9 (±12.2) |

[a]Each insect tested weighed between 339 and 45 3 mg. Extraction solvent was methylene chloride.
[b]Analysis of variance showed no differences among means ($F[3, 36] = 0.19$, $P > 0.90$).

were essential, together with the contents of the Stobbe glands, for the production of the male pheromone. It is possible that for this species the enzyme responsible for glycoside degradation is produced by the pocket scales.

Given this information concerning male pheromone production, there are two possible explanations for the absence of benzaldehyde in the male having partially exposed hairpencils. Firstly, if the male failed to retract his hairpencils at emergence, they may never have come into contact with the pheromone. Grant et al. (1972) observed that hairpencils of newly emerged *P. unipuncta* males contained no pheromone constituents and, as an explanation, stated that the hairpencils were empty "probably because they were not contained in their abdominal pockets or had not been in them long enough." It is possible that the retraction of the hairpencils is in some way necessary in the initial pheromone production. While this was not directly discussed by Birch (1970b), he did report that the Stobbe glands of *P. meticulosa* only discharged following the first in-

TABLE 3. AMOUNT OF BENZALDEHYDE/INSECT FROM HAIRPENCILS OF 4-DAY-OLD VIRGIN *Pseudaletia unipuncta* MALES, EXCISED AT DIFFERENT TIMES DURING A 16:8 L:D PHOTOPERIOD

| Time | Number of insects[a] | $\bar{X}$ benzaldehyde/insect ($\mu g \pm SD$)[b] |
|---|---|---|
| 1 hr after scotophase onset | 10 | 34.2 (±11.6) |
| 7 hr after scotophase onset | 10 | 42.9 (±5.6) |
| 5 hr after photophase onset | 10 | 37.0 (±11.4) |

[a]Each insect tested weighed between 305 and 431 mg. Extraction solvent was methylene chloride.
[b]Analysis of variance showed no differences among means ($F[2,27] = 0.19$, $P > 0.90$).

TABLE 4. AMOUNT OF BENZALDEHYDE FROM INDIVIDUAL HAIRPENCILS OF 4-DAY-OLD VIRGIN *Pseudaletia unipuncta* MALES[a]

| Benzaldehyde/hairpencil ($\mu$g ± SD) | | Benzaldehyde/hairpencil (% of total) | |
|---|---|---|---|
| Left | Right | Left | Right |
| 23.6 (±0.3) | 16.0 (±0.1) | 59.6 | 40.4 |
| 5.6 (±0.3) | 8.5 (±0.1) | 39.4 | 60.6 |
| 9.9 (±0.3) | 8.0 (±0.1) | 56.2 | 43.8 |
| 27.0 (±0.2) | 27.2 (±0.3) | 49.8 | 50.2 |
| 11.9 (±0.1) | 0.0 | 100.0 | 0.0 |
| 18.1 (±0.1) | 16.8 (±0.1) | 51.9 | 48.1 |
| 14.5 (±0.1) | 10.6 (±0.1) | 57.1 | 42.9 |
| 0.0 | 29.9 (±0.1) | 0.0 | 100.0 |
| 28.1 (±0.3) | 20.3 (±0.2) | 58.1 | 41.9 |
| 11.2 (±0.1) | 15.1 (±0.2) | 42.6 | 57.4 |

[a]Data from 10 insects each weighing between 346 and 399 mg. Extraction solvent was methylene chloride.

sertion of the hairpencils into the pockets. The second explanation is that while the hairpencils had initially been drawn into the pockets at emergence, all benzaldehyde present was lost through evaporation from the exposed distal tips.

Data presented in Table 4 show that the difference between the amount of benzaldehyde found in the left and right hairpencils from the same individual varies considerably. In two of the 10 males examined, 100% of the benzaldehyde was contained in one hairpencil. The "empty" hairpencils appeared to have been completely retracted, as neither was noticeably everted or exposed. Even if one hairpencil is empty, the other may contain a large amount of benzaldehyde, e.g., one male, having no benzaldehyde in the left hairpencil, had 29.9 $\mu$g in the right. This is the highest amount recorded in a single hairpencil from the 10 males examined and is approximately equal to the mean benzaldehyde/insect value of 30.2 ± 13.4 $\mu$g ($\bar{X}$ ± SD). Toth (1982) showed that *Mamestra brassicae* (L.) males with one hairpencil excised are able to mate successfully, indicating "one-sided" males are reproductively functional. Whether they are competitive with normal males, however, remains to be determined.

As none of the treatments significantly affected the yield of benzaldehyde/insect, we pooled results from all experiments in order to examine the relationship between pupal weight and benzaldehyde which, when plotted as a percentage of pupal weight, is approximately normally distributed (Figure 1). A simple linear regression analysis of benzaldehyde on pupal weight, although significant ($P > 0.01$), indicated that pupal weight is associated with only 8% of the

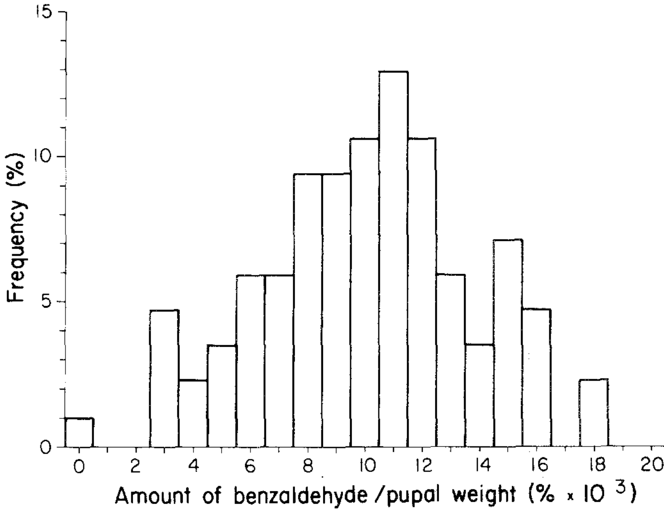


FIG. 1. Distribution of amounts of benzaldehyde from 4-day-old virgin *Pseudaletia unipuncta* males. Amounts are expressed as percentages of pupal weight ($\times 10^3$). $N = 85$.

variation in benzaldehyde ($r^2 = 0.076$). We also examined the relationships between hairpencil length and pupal weight on the quantity of benzaldehyde/insect. As hairpencils often break during excision or measurement, only 52 of the 84 insects could be used in the analyses. Simple linear regressions of mean hairpencil length/insect on pupal weight ($P > 0.99$) and of the amount of benzaldehyde/insect on mean hairpencil length/insect ($P > 0.68$) were not significant. Therefore, neither pupal weight nor hairpencil length are useful parameters for estimating the quantity of pheromone present in *P. unipuncta* males.

Yields of benzaldehyde/insect obtained from the sample of 84 males ranged from 10.5 to 72.3 $\mu$g, with a mean of 38.6 $\pm$ 13.9 $\mu$g ($\bar{X} \pm$ SD). These values are, on average, higher than previously reported maxima of 45.6 $\mu$g (Grant et al., 1972) and 22.4 $\mu$g (Hirai, 1980).

Some benzaldehyde is inevitably lost as hairpencils are excised and transferred to the extraction vial (one often smells the almond-like odor of benzaldehyde); this loss is minimized by proceeding as quickly as possible and by keeping the vials chilled. Benzaldehyde may also be lost if the distal portion of the hairpencil breaks during excision. Extraction of distally broken hairpencils could therefore underestimate amounts of benzaldehyde and, in young males, overestimate the ratio of benzyl alcohol to benzaldehyde.

The results of the experiments presented herein clearly demonstrate that the excision and extraction techniques tested do not influence the quantities of benzaldehyde recovered from single *P. unipuncta* virgin males and that the observed differences reflect biological variability between individuals. However, in order to standardize procedures in all future experiments, individual males will

be immobilized by chilling during the last hour of the scotophase, their hairpencils excised and, whenever possible, the extracts analyzed within 72 hr.

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# ATTRACTION OF TORTRICID MOTHS OF SUBFAMILY OLETHREUTINAE TO FIELD TRAPS BAITED WITH DODECADIENES

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**Abstract**—All four geometrical isomers of 7,9- and 8,10-dodecadienes with acetate, alcohol, and aldehyde functional groups were synthesized and field tested. The field survey produced sex attractant lures for 14 insect species. Species in the genera *Cydia, Grapholita, Eucosma, Pelochrista, Petrova, Phenta, Hedya,* and *Pseudosciaphila* were captured. Defined lures were developed for some of the species captured. Gas chromatographic retention times for all geometrical isomers of 7,9- and 8,10-dodecadienes with acetate, alcohol, and aldehyde functional groups are reported. A study of the isomerization of 8,10-dodecadienyl acetates and aldehydes impregnated in rubber septa is reported.

**Key Words**—Sex attractant, Lepidoptera, Tortricidae, Olethreutinae, 8,10-dodecadienes, 7,9-dodecadienes, field trapping.

## INTRODUCTION

Two different approaches have been used to discover sex attractant chemicals for lepidopterous insects. The classical method is to solvent extract the pheromone glands of virgin female moths and chemically identify the attractant from the extract. Subsequent synthesis and positive field tests confirm the isolated chemicals are part of the insects' sex pheromone. The second method is to field screen pheromone-like synthetic chemicals. Using this method, the chemical structure is known but no particular species is targeted. Many new sex attractants for moths in the families Noctuidae and Tortricidae have been discovered by



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field screening (e.g., Steck et al., 1977, 1979, 1982; Ando et al., 1977, 1978, 1981). While sex attractants found by field screening do not necessarily correspond with the natural pheromone, they can serve as a guide to possible chemical structure when isolation from a particular species is undertaken.

The chemical structure–activity relationship for sex attractants in the Tortricidae has been reviewed (Roelofs and Brown, 1982). All sex attractant chemicals have been straight-chain alcohols, acetates, or aldehydes with one or two double bonds in the carbon chain. With few exceptions the subfamily Tortricinae respond to chemicals with 14-carbon chain lengths and the subfamily Olethreutinae respond to chemicals with 12-carbon chain lengths. Of 66 species of Olethreutinae reported, 13 were attracted to conjugated dienes: (8*E*,10*E*)-8,10-dodecadienyl acetate (8*E*,10*E*–12 : Ac) attracted eight, (8*E*,10*Z*)-8,10-dodecadienyl acetate (8*E*,10*Z*-12 : Ac) + 8*E*,10*E*-12 : Ac attracted one, (8*E*,10*E*)-8,10-dodecadien-1-ol (8*E*,10*E*-12 : OH) attracted one, (7*E*,9*Z*)-7,9-dodecadienyl acetate (7*E*,9*Z*-12 : Ac) attracted one, (9*E*,11)-9-11-dodecadienyl acetate (9*E*,11–12 : Ac) attracted one, and (8*Z*,10*Z*)-8,10-hexadecadienyl acetate (8*Z*,10*Z*-16 : Ac) attracted one. Additionally, Hathaway and Tamaki (1981) have reported attraction of *Phaneta latens* to traps baited with 8*E*,10*E*-12 : OH.

We report here the results of a field survey of some conjugated dienes with 12 carbons and terminal alcohol, acetate, or aldehyde functional groups. Chemical attractants not previously reported were found for 14 species of Olethreutinae. We also report partly defined baits for some of the species which in some cases include chemical synergists or inhibitors.

## METHODS AND MATERIALS

Field trapping was carried out 100 km northeast of Saskatoon, Canada, in a forest area which contained spruce, pine, birch, and aspen trees, and a variety of herbaceous shrubs. The area also contained an occasional small meadow. Pherocon 1CP traps (Zoecon Corp., Palo Alto, California) containing the chemical lures impregnated into rubber septa (Authur H. Thomas No. 8753-D22) were hung on tree branches. Each lure was routinely protected by adding 2 drops of a 10% solution of the antioxidant butylated hydroxytoluene (BHT) in acetone to the septa. The traps, 1–2 m above the ground and spaced 15–20 m apart, were inspected weekly; captures were recorded and sticky liners were replaced as required.

The chemicals used in this study were synthesized and purified in this laboratory. The 8,10-dodecadienes and the 7,9-dodecadienes with alcohol, acetate, or aldehyde functions were synthesized by procedures previously described for the synthesis of the 5,7-dodecadienes (Chisholm et al., 1981, Palaniswamy et al., 1983). All four geometrical isomers of the 7,9-dodecadien-1-ols were recovered from Wittig condensation reactions: the *E,Z* and *E,E,* isomers from condensation of the ylide of propyltriphenylphosphonium bromide with (*E*)-9-

tetrahydropyranyloxy-2-nonenal, the *Z,Z* and *E,E* isomers from condensation of the ylide of (*Z*)-2-pentenyltriphenylphosphonium bromide with 7-acetoxyheptanal, the *Z,E* and *E,E* isomers from condensation of the ylide of 7-acetoxyheptyltriphenylphosphonium bromide with (*E*)-2-pentenal (Palaniswamy et al., 1983).

Three geometrical isomers of 8,10-dodecadien-1-ols were recovered from Wittig condensation reactions: the *E,Z* and *E,E* isomers from condensation of the ylide of ethyltriphenylphosphonium bromide with (*E*)-10-tetrahydropyranyloxy-2-decenal, the *Z,E* and *E,E* isomers from condensation of the ylide of 8-acetoxyoctyltriphenylphosphonium bromide with (*E*)-2-butenal.

The (8Z,10Z)-8,10-dodecadien-1-ol (8Z,10Z-12:OH) was obtained by synthesis and stereoselective reduction of 8,10-dodecadiyn-*tert*-butyldimethylsilyl ether (Figure 1).

*8,10-Dodecadiyn-1-ol, III.* To a well-stirred solution of cuprous chloride (0.06 g, 0.61 mmol) and hydroxylamine hydrochloride (0.12 g, 1.73 mmol) in 4.4 ml of 70% ethylamine and 3 ml of water at 0°C was added dropwise 8-nonyn-1-ol I (Abrams, 1984) (2.8 g, 20 mmol) over 30 min. Then 1-bromopropyne II (2.3 g, 20 mmol) was added dropwise to the reaction. After stirring 16 hr at 21°C, potassium cyanide (0.25 g, 3.85 mmol) was added to the blue–green reaction (DeGraw and Rodin, 1971). The resulting orange-colored solution was added to 50 ml of water and the product extracted with 3 × 30 ml of ether. The extract was washed with 30 ml of water and dried. Evaporation of the ether gave 2.14 g, 12 mmol III.

*8,10-Dodecadiyn-Tert-Butyldimethylsilyl ether, IV. tert*-Butyldimethylsilyl chloride (1.0 g, 6.64 mmol) was added to a well stirred solution of imidazole (1.0 g, 14.7 mmol) and III (0.98 g, 5.5 mmol) in DMF (6 ml) at 0°C. After warming to 21°C and stirring for 1 hr, the reaction mixture was poured into water (25 ml) and the product IV was extracted with 3 × 25 ml of pentane.

FIG. 1. Stereoselective synthesis of (8Z,10Z)-8,10-dodecadien-1-ol.

The extract was dried and the solvent and silyl alcohol were evaporated, 1 torr 40°C.

*(8Z,10Z)-8,10-Dodecadien-tert-butyldimethylsilyl ether, V.* Borane–methylsulfide complex 10.0 M in $BH_3$ (13 mmol) (Aldrich Chemical Co.) was slowly added under argon to a stirred solution of cyclohexene (2.13 g, 26 mmol) (Zweifel and Polston, 1970) in 3 ml of dry THF at 0°C. After 1 hr, IV (1.6 g, 5.5 mmol) in THF (5 ml) was added and the reaction warmed to 21°C and stirred for 18 hr. Glacial acetic acid (2.5 ml) was added and the reaction was stirred 4 hr at 55–60°C. The borane was treated with 11 ml of 6N NaOH followed by dropwise addition of 30% hydrogen peroxide (2.5 ml); temperature maintained below 35°C. After stirring for 30 min, brine (5 ml) was added and the product was extracted with pentane (3 × 25 ml). The extract was washed with water and after drying the solvent was evaporated. Yield 1.13 g, 3.8 mmol V.

(8Z,10Z)-8,10-Dodecadien-1-ol, VI. The *tert*-butyldimethylsilyl protecting group was removed from V (1.13 g, 3.8 mmol) by stirring with tetrabutylammonium fluoride (10 ml of 1 M THF sol). After 2 hr, water (25 ml) was added and the product was extracted with pentane (3 × 25 ml). The extract was washed with water and dried. The solvent and silyl alcohol were evaporated at 1 torr. The product was purified by flash chromatography (Still et al., 1978) from Kieselgel 60 using a solvent gradient of hexane to ethyl acetate (increasing by 5% per liter), followed by argentation chromatography (Houx et al., 1974). The product 0.553 g, 3.0 mmol was 99% pure by GC analysis and gave expected NMR and IR spectra. (8Z,10Z)-8,10-Dodecadienyl acetate (8Z,10Z-12 : Ac) was prepared by treating 8Z,10Z-12 : OH with hot acetic anhydride. (8Z,10Z)-8,10-Dodecadienal (8Z,10Z-12 : Ald) was prepared by oxidation of 8Z,10Z-12 : OH with pyridinium chlorochromate (Corey and Suggs, 1975).

*Dodecenes.* The monoenes were synthesized by Wittig reaction: condensation of the ylide of butyltriphenylphosphonium bromide with acetoxyoctanal gave (*E*)- and (*Z*)-8-dodecenyl acetate (Z8–12 : Ac) and condensation of the ylide of ethyltriphenylphosphonium bromide with acetoxydecanal gave (*E*)- and (*Z*)-10-dodecenyl acetate (Z10–12 : Ac). The acetate groups were removed and the *E* and *Z* alkenols were separated using argentation chromatography. The acetates were regenerated by treating a portion of the alkenols with hot acetic anhydride.

Geometrical isomerization of 8,10-dodecadienes in the releasers was determined by weathering the rubber septa loaded with specific isomers outdoors. After various periods of time, the septa were extracted (Butler and McDonough, 1979) and the isomeric distribution was determined by GC analysis.

Chromatographic analyses were performed on a Hewlett-Packard gas chromatograph with flame-ionization detector. All four geometrical isomers of 7,9- and 8,10-dodecadienes with alcohol, acetate, or aldehyde functions were separated on a DB-5 fused silica capillary column (0.32 mm ID × 30 m; J and W Scientific Inc., Rancho Cardova, California) programmed 90°–200°C at 4°/min. Their retention times relative to *nd*-tridecyl acetate are reported in Table 8.

Data from the replicated field tests were transformed $\sqrt{x + 1}$ and then analyzed by an analysis of variance; significantly different means were separated by Duncan's multiple-range test.

## RESULTS AND DISCUSSION

Table 1 shows tortricids captured in the forest survey with dienes. All four geometrical isomers of 8,10- and 7,9-dodecadienes with alcohol, acetate, or aldehyde functions were tested. As expected, most multiple insect captures were from the subfamily Olethreutinae. Most species were attracted to various 8,10-dodecadienes, one species was attracted to 7,9-dodecadienes, and one species, *Cydia flexiloqua*, was attracted to traps baited for *Malacosoma disstria* (Chisholm et al., 1982) containing a mixture of 5,7-dodecadienes and (*Z*)-7-dodecenal (*Z*7–12 : Ald). Sex attractants have been reported for two species listed in Table 1: *G. prunivora* (Roelofs and Carde, 1974) and *H. ochroleucana* (Frerot et al., 1979). We are not aware of sex attractant reports for the remaining 12 moths, two of which are undescribed (Dr. P.T. Dang, personal communication).

After multiple captures of a single species were recorded in survey traps, a 3× replicated test was set in the field. Some species were attracted to more than one survey chemical. Chemicals for the multiple component lures, in the replicated tests, were selected on the basis of captures by the survey and on a "one change" in chemical structure (Steck et al., 1982): a chemical was combined with analogs differing either in the terminal functional group or the geometry of the double bonds.

Tables 2–7 present trapping data from some of the replicated field trials carried out in 1982 or 1983.

In 1982 *G. lunatana* was captured by traps baited with 8*E*,10*E*-12 : Ac. Subsequently, the 8*E*,10*E*-12 : Ac was tested in combination with many other 8,10-dienes and with *E*- and *Z*8–12 : OH and *E*- and *Z*10–12 : OH and their acetates. Various combinations of the monoenes alone were also tested. No combination caught significantly more *G. lunatana* than the 8*E*,10*E*-12 : Ac alone, but captures were reduced substantially when *Z*8–12 : Ac was the second component. In addition, an undescribed *Grapholita* sp. (Table 2) attracted by *E*8–12 : Ac was inhibited when *Z*8–12 : Ac, 8*Z*,10*Z*-12 : Ac or 8*E*,10*E*-12 : Ac was added to the lure. The 1982 field trapping data for *G. lunatana* were confirmed and extended in 1983 (Table 3). Six compounds (8*E*,10*E*-12 : OH, 8*E*,10*Z*-12 : Ac, 8*Z*,10*E*-12 : Ac, 8*Z*,10*Z*-12 : Ac, *Z*10–12 : Ac and *E*8–12 : Ac) were mildly inhibitory and one compound (*Z*8–12 : Ac) was a potent inhibitor of *G. lunatana* when added to the sex attractant 8*E*,10*E*-12 : Ac. Table 3 also shows *C. ingrata* was lured to traps baited with 8*E*,10*E*-12 : Ac + 8*E*,10*Z*-12 : Ac. *C. ingrata* probably required the two-component lure as it was not captured by a survey trap baited with 8*E*,10*Z*-12 : Ac alone. In a separate test (Table 4) near Saskatoon, *G. pru-*

TABLE 1. TORTRICID MOTHS CAPTURED BY SYNTHETIC SEX ATTRACTANTS

| Tortricidae: Olethreutinae | Chemicals | Component ratio | No. trapped | Flight period |
|---|---|---|---|---|
| Tribe Laspeyresiini | | | | |
| *Cydia flexiloqua* (Heinr.) | 5*Z*,7*E*-12:Ald + 5*Z*,7*Z*-12:Ald + *Z*7-12:Ald | 100:10:10 | 34 | 7 July–25 July |
| *C. lautiuscula* (Heinr.) | 8*E*,10*E*-12:Ac | 100 | 86 | 7 June–29 June |
| *C. populana* (Busck) | 8*E*,10*E*-12:Ald | 100 | 953 | 21 June– 3 Aug |
| *C. toreuta* | 8*E*,10*Z*-12:Ac | 100 | 29 | 10 June–30 June |
| *C. ingrata* (Heinr.) | 8*E*,10*E*-12:Ac + 8*E*,10*Z*-12:Ac | 100:10 | 118 | 16 May–13 June |
| *Grapholita lunatana* (Wlshm.) | 8*E*,10*E*-12:Ac | 100 | 3320 | 16 May–26 June |
| *G.* species (undescribed)[a] | 8*E*,10*E*-12:Ald + 8*E*,10*E*-12:Ac | 100:10 | 304 | 5 July– 3 Aug |
| *G. prunivora* (Walsh.) | *Z*8–12:Ac + *E*8–12:Ac | 200:5 | 390 | June– Aug |
| *G.* species (undescribed)[a] | *E*8–12:Ac | 100 | 340 | 2 June–29 June |
| Tribe Eucosmini | | | | |
| *Eucosma delericta* (Heinr.) | 7*E*,9*E*-12:Ac + 7*Z*,9*E*-12:Ac | 100:100 | 11 | 3 Aug–10 Aug |
| *Pelochrista scintillana randana* (Kft.) | 8*E*,10*E*-12:Ac + *Z*8–12:Ac + *E*8–12:Ac | 10:200:5 | 11 | 27 July–19 Aug |
| *Petrova burkeana* (Kft.) | 8*E*,10*E*-12:Ac | 100 | 206 | 5 July–27 July |
| *Phaneta ochroterminana* (Kft.) | 8*E*,10*E*-12:Ac + 8*Z*,10*Z*-12:Ac | 10:100 | 76 | 20 July–10 Aug |
| *P. roseoterminana* (Kft.) | 8*Z*,10*Z*-12:OH | 100 | 12 | 21 June– 5 July |
| Tribe Olethreutini | | | | |
| *Hedya ochroleucana* (Hbn.) | 8*Z*,10*E*-12:Ac + 8*Z*,10*Z*-12:Ac | 100:10 | 287 | 7 July– 5 Aug |
| *Pseudosciaphila duplex* (Wlsm.) | 8*Z*,10*E*-12:Ac + 8*Z*,10*E*-12:Ald | 100:100 | 665 | 30 June–18 July |

[a] Undescribed *Grapholita* sp. Biosystematic Research Institute (Agriculture Canada).

TABLE 2. RESPONSE OF *Grapholita lunatana* AND UNDESCRIBED *Grapholita* SP. TO 8,10-DODECADIENES AND 8- AND 10-DODECENES

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | *G. lunatana* | *Gropholita* sp.[b] |
| 8*E*,10*E*-12 : Ac (100) | 85a | 0 |
| 8*E*,10*E*-12 : Ac (100 + 8*Z*,10*Z*-12 : Ac (10) | 36ab | 0 |
| 8*E*,10*E*-12 : Ac (100) + *Z*8-12 : Ac (10) | 6c | 0 |
| 8*E*,10*E*-12 : Ac(100) + 8*Z*,10*E*-12 : Ac(10) | 32ab | 0 |
| *E*8-12 : Ac (50) + *E*10-12 : Ac (50) | 0 | 27 ab |
| *E*8-12 : Ac (100) + *Z*8-12 : Ac (10) | 0 | 9 bc |
| *E*8-12 : Ac (100) + 8*E*-12 : OH (10) | 0 | 36 a |
| *E*8-12 : Ac (100) + 8*E*,10*E*-12 : Ac (10) | 0 | 8 bc |
| *E*8-12 : Ac (100) + 8*Z*,10*Z*-12 : Ac (10) | 0 | 1 c |
| *E*8-12 : Ac (10) | 0 | 30 ab |
| *E*8-12 : Ac (100) | 0 | 45 a |

[a]3× replicated, 9 June to 15 June 1982. Numbers followed by the same letter are not different (P=0.05)
[b]Undescribed *Grapholita* sp. Biosystematics Research Institute, (Agriculture Canada)

*nivora* was captured by traps baited with the lure *Z*8–12:Ac (200 μg) + *E*8–12:Ac (5 μg) (Roelofs and Carde, 1974). We found adding 8*E*,10*E*-12:Ac to that lure inhibited capture of *G. prunivora.* The data in Tables 2, 3, and 4 suggest species isolation may, in part, be accomplished by a combination of monenes and dienes in the chemical communications system of some *Grapholita* species.

A replicated field test followed capture of *Cydia populana* and an undescribed *Grapholita* species by a single survey trap baited with 8*E*,10*E*–12:Ald. Table 5 presents the results of that test. Only one of the five additive compounds tested significantly changed the capture rate of *C. populana.* Combining 8*E*,10*E*-12:Ac with 8*E*,10*E*-12:Ald markedly reduced the number of *C. populana* attracted to the traps, but the same lure greatly increased capture of the *Grapholita* species. While 8*E*,10*E*-12:Ac alone was not included in this test, neither moth species was attracted by survey traps baited with 8*E*,10*E*-12:Ac.

Table 6 presents field trapping data for *Pseudosciaphila duplex* and *Hedya ochroleucana*. Of the various chemical combinations tested, 8*Z*,10*E*-12:Ac + 8*Z*,10*E*-12:Ald in 1:1 ratio was the best attractant for *P. duplex* and 8*Z*,10*E*-12:Ac + 8*Z*,10*Z*-12:Ac in 10:1 ratio was the best attractant for *H. ochroleucana*. When small amounts of 8*Z*,10*Z*-12:Ac were added to the lure composed of 8*Z*,10*E*-12:Ac + 8*Z*,10*E*-12:ALD, *P. duplex* was inhibited and *H. ochroleucana* was attracted to the lure. Frerot et al. (1979) have reported attraction of *H. ochroleucana* to 8*Z*,10*E*-12:Ac alone. Table 6 shows adding 8*Z*,10*Z*-12:Ac

TABLE 3. CAPTURE OF *Grapholita lunatana* AND *Cydia ingrata* BY TRAPS BAITED WITH 8,10-DODECADIENES

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | *G. lunatana* | *C. ingrata* |
| 8*E*,10*E*-12 : Ac(100) | 445 ab | 1 b |
| 8*E*,10*E*-12 : Ac(100) + 8*Z*,10*E*-12 : OH(10) | 479 a | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*E*,10*E*-12 : OH(10) | 144 d | 1 b |
| 8*E*,10*E*-12 : Ac(100) + 8*E*,10*E*-12 : Ald(10) | 376 abc | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*E*,10*Z*-12 : Ac(10) | 237 bcd | 118 a |
| 8*E*,10*E*-12 : Ac(100) + 8*Z*,10*E*-12 : Ac(10) | 200 cd | 2 b |
| 8*E*,10*E*-12 : Ac(100) + 8*Z*,10*Z*-12 : Ac(10) | 228 bcd | 3 b |
| 8*E*,10*E*-12 : Ac(100) + *Z*8-12 : Ac(10) | 20 e | 0 b |
| 8*E*,10*E*-12 : Ac(100) + *Z*10-12 : Ac(10) | 236 cd | 0 b |
| 8*E*,10*E*-12 : Ac(100) + *E*8-12 : Ac(10) | 211 cd | 0 b |
| 8*E*,10*E*-12 : Ac(100) + *E*10-12 : Ac(10) | 393 abc | 0 b |
| 8*E*,10*E*-12 : Ac(100) + *E*10-12 : OH(10) | 274 abcd | 0 b |

[a]3× replicated, May 16, to June 13, 1983. Numbers followed by the same letter are not different (P = 0.05)

to 8*Z*,10*E*-12 : Ac increases the capture of *H. ochroleucana* by more than 12-fold. The data in Table 6 show a possible role for chemical attractants in species isolation.

Table 7 presents results from a replicated test set in the field after *Petrova burkeana* was captured by a survey trap baited with 8*E*,10*E*-12 : Ac. All second-component dienes added to 8*E*,10*E*-12 : Ac in 1 : 10 ratio significantly reduced *P. burkeana* captures, except for 8*Z*,10*Z*-12 : Ac which had no effect. However, when 8*Z*,10*Z*-12 : Ac was combined with 8*E*,10*E*-12 : Ac in 10 : 1 ratio, *P. bur-*

TABLE 4. INHIBITION OF *Grapholita prunivora* BY (8*E*,10*E*)-8,10-DODECADIEN-1-OL ACETATE

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | *G. prunivora* | *P. scintillana randana* |
| *Z*8-12 : Ac(200) + *E*8-12 : Ac(5) | 186 a | 0 |
| *Z*8-12 : Ac(200) + *E*8-12 : Ac(5) + 8*E*,10*E*-12 : Ac(100) | 4 b | 0 |
| *Z*8-12 : Ac(200) + *E*8-12 : Ac(5) + 8*E*,10*E*-12 : Ac(10) | 15 b | 11 |

[a]3× replicated, June 30, to Aug. 19, 1982. Numbers followed by the same letter are not different (*P* = 0.05).

TABLE 5. CAPTURE OF *Cydia populana* AND A *Grapholita* SP. BY TRAPS BAITED WITH 8,10-DODECADIENES

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | *C. populana* | *Grapholita* sp.[a] |
| 8*E*,10*E*-12 : Ald(100) | 250 a | 27 b |
| 8*E*,10*E*-12 : Ald(100) + 8*E*,10*E*-12 : OH(10) | 153 a | 5 c |
| 8*E*,10*E*-12 : Ald(100) + 8*E*,10*E*-12 : Ac(10) | 6 b | 268 a |
| 8*E*,10*E*-12 : Ald(100) + 8*Z*,10*Z*-12 : Ald(10) | 119 a | 3 c |
| 8*E*,10*E*-12 : Ald(100) + 8*Z*,10*E*-12 : Ald(10) | 152 a | 0 c |
| 8*E*,10*E*-12 : Ald(100) + 8*E*,10*Z*-12 : Ald(10) | 273 a | 1 c |

[a]3× replicated, June 21, to August 3, 1982. Numbers followed by the same letter are not different ($P = 0.05$).
[b]Undescribed *Grapholita* sp. Biosystematics Research Institute (Agriculture Canada).

*keana* was excluded and *Phaneta ochroterminana* was attracted. *P. ochroterminana* was not captured by survey traps baited with 8Z,10Z-12 : Ac alone.

The insect species listed in Table 1 were attracted by chemicals with $C_{12}$ chains. All were from the subfamily Olethreutinae, in accord with the previous observation (Roelofs and Brown, 1982) that Olethreutinae are attracted primarily by 12-carbon compounds. Four of the species listed in the Table 1 were attracted to chemicals with aldehyde functions. We are not aware of any literature report of Olethreutinae being attracted by aldehydes. The *Cydia* species in Table 1 attracted by the 8,10-dodecadienes are narrowly sympatric, and therefore sex attractant chemicals may play a role in species isolation.

This investigation has provided sex lures that could be used to detect the presence of, or study the habits of, *P. duplex, H. ochroleucana, P. burkeana, P. ochroterminana, C. populana, C. ingrata,* and *G. lunatana*. For best attraction, two-component lures are required by four of the foregoing species. But the best attractant found for *G. lunatana*, 8*E*,10*E*-12 : Ac alone, was inhibited by *Z*8–12 : Ac. *G. prunivora,* attracted by a combination of *Z*- and *E*8–12 : Ac, was inhibited by 8*E*,10*E*-12 : Ac. Lures composed of diene and monoene combinations may be required for optimum capture or isolation of some species.

Both *Hedya nubiferana* (Frerot et al., 1979) and *Hedya chionosema* (Roelofs and Brown, 1982) are attracted by diene–monoene combinations. To date, the best attractant reported for *Cydia pomonella* (codling moth) is 8*E*,10*E*-12 : OH. However, Bartell and Bellas (1981), working with GC fractions from female abdominal tip extract and synthetic 8*E*,10*E*-12 : OH, have shown in laboratory bioassay that *C. pomonella* pheromone probably has more than one component. Perhaps a diene–monoene combination would be effective.

The stereochemical purity of 8Z,10Z-12 : OH (Figure 1) was better than

TABLE 6. CAPTURE OF *Pseudosciaphila duplex* AND *Hedya ochroleucana* BY TRAPS BAITED WITH 8,10-DODECADIENES

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | *P. duplex* | *H. ochroleucana* |
| 8*Z*,10*E*-12: Ac(100) | 14 cde | 12 de |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(10) | 95 b | 63 b |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(100) | 191 a | 65 bc |
| 8*Z*,10*E*-12: Ac(10) + 8*Z*,10*E*-12: Ald(100) | 45 bc | 14 de |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(1) | 49 bc | 19 cde |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(100) + 8*Z*,10*Z*-12: Ac(10) | 15 cde | 148 a |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(100) + 8*Z*,10*E*-12: OH(10) | 67 b | 26 bcd |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(100) + 8*E*,10*E*-12: OH(10) | 66 b | 31 bcd |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*E*-12: Ald(100) + 8*E*,10*Z*-12: Ac(10) | 197 a | 20 cde |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*Z*-12: Ac(10) | 4 e | 179 a |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*Z*-12: Ac(100) | 0 e | 15 cde |
| 8*Z*,10*E*-12: Ac(10) + 8*Z*,10*Z*-12: Ac(100) | 0 e | 0 e |
| 8*Z*,10*E*-12: Ac(100) + 8*Z*,10*Z*-12: Ac(100) | 41 bcd | 6 de |
| 8*Z*,10*Z*-12: Ac(100) | 0 e | 1 e |
| 8*Z*,10*Z*-12: Ac(100) + 8*Z*,10*E*-12: Ald(10) | 0 e | 0 e |
| 8*Z*,10*E*-12: Ald(100) | 10 de | 0 e |

[a]3× replicated, June 30, to August 2, 1983. Numbers followed by the same letter are not different ($P = 0.05$)

TABLE 7. CAPTURE OF *Petrova burkeana* AND *Phaneta ochroterminana* BY TRAPS BAITED WITH 8,10-DODECADIENES

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | *P. burkeana* | *P. ochroterminana* |
| 8*E*,10*E*-12 : Ac(100) | 57 a | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*E*,10*E*-12 : OH(10) | 1 b | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*E*,10*E*-12 : Ald(10) | 15 b | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*E*,10*Z*-12 : Ac(10) | 6 b | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*Z*,10*E*-12 : Ac(10) | 15 b | 0 b |
| 8*E*,10*E*-12 : Ac(100) + 8*Z*,10*Z*-12 : Ac(10) | 59 a | 0 b |
| 8*E*,10*E*-12 : Ac(10) + 8*Z*,10*Z*-12 : Ac(100) | 0 b | 76 a |

[a]3× replicated, 15 July to 10 August 1982. Numbers followed by the same letter are not different ($P = 0.05$).

98%. The critical step in the synthesis, reduction of the diyne, has been reported for other compounds (Sonnet and Heath, 1980), but we are not aware of any report of 8Z,10Z-12:OH being prepared using this synthetic route.

The elution order of positional isomers of the conjugated dodecadienes from the DB-5 column was different (Table 8). The elution order of 8,10-dodecadienes was *ZE, EE, EZ, ZZ,* but the 7,9-dodecadienes eluted *ZE, EZ, ZZ, EE.*

Conjugated dienes are known to isomerize under field conditions (Shani and Klug, 1980: Ideses et al., 1982). This lack of stability in the geometrical isomers can affect trap capture of insects when the geometrical isomers are used as lures

TABLE 8. RELATIVE RETENTION TIMES OF GEOMETRICAL ISOMERS OF 8,10- AND 7,9-DODECADIENYL ALDEHYDES, ALCOHOLS, AND ACETATES RELATIVE TO *n*-TRIDECYL ACETATE (1,000 = 20.88 min)[a]

| Isomers | Aldehydes | Alcohols | Acetates |
|---|---|---|---|
| 8,10-Dodecadienes | | | |
| (*Z*,*E*) | 0.6489 | 0.7423 | 0.9272 |
| (*E*,*E*) | 0.6633 | 0.7557 | 0.9411 |
| (*E*,*Z*) | 0.6710 | 0.7625 | 0.9478 |
| (*Z*,*Z*) | 0.6748 | 0.7668 | 0.9526 |
| 7,9-Dodecadienes | | | |
| (*Z*,*E*) | 0.6341 | 0.7261 | 0.9119 |
| (*E*,*Z*) | 0.6456 | 0.7371 | 0.9229 |
| (*Z*,*Z*) | 0.6537 | 0.7452 | 0.9320 |
| (*E*,*E*) | 0.6595 | 0.7514 | 0.9387 |

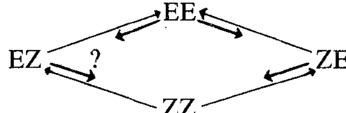
[a]On J and W Scientific DB-5 (0.32 mm ID × 30 m) temperature programmed from 90° to 200° C at 4°/min.

TABLE 9. ISOMERIZATION OF 8,10-DODECADIENYL ACETATE AND 8,10-DODECADIENAL IN RUBBER SEPTA AFTER VARIOUS PERIODS OF TIME

| | Acetates | | | | | Aldehydes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | % isomerism[b] | | | | | % isomerism[b] | | | |
| Time days | Total μg remaining[a] | EE | EZ | ZE | ZZ | Total μg remaining[a] | EE | EZ | ZE | ZZ |
| 0 | 242.0 | 100.0 | — | — | — | 236.9 | 98.8 | 1.2 | — | — |
| 7 | 159.7 | 98.1 | — | 1.9 | — | 67.1 | 81.7 | 16.2 | 2.1 | — |
| 14 | 138.0 | 95.1 | — | 4.9 | — | 41.1 | 86.6 | 9.3 | 4.1 | — |
| 25 | 102.3 | 95.5 | — | 4.5 | — | 30.5 | 100.0 | — | — | — |
| 0 | 263.0 | — | 100.0 | — | — | 214.6 | 0.8 | 98.4 | 0.8 | — |
| 7 | 197.2 | 8.4 | 89.1 | 2.5 | — | 63.0 | 4.3 | 91.6 | 4.1 | — |
| 14 | 165.4 | 14.8 | 81.4 | 3.8 | — | 47.6 | 4.0 | 96.0 | — | — |
| 25 | 107.4 | 25.0 | 69.2 | 5.8 | — | 29.6 | 13.2 | 86.8 | — | — |
| 0 | 320.0 | 0.8 | 0.4 | 98.5 | 0.3 | 212.6 | — | 0.3 | 99.7 | — |
| 7 | 191.1 | 5.8 | 1.8 | 90.5 | 1.9 | 63.5 | 5.0 | 1.1 | 93.9 | — |
| 14 | 156.3 | 9.3 | 1.9 | 86.4 | 2.4 | 44.9 | 4.2 | 5.4 | 83.5 | 6.9 |
| 25 | 126.1 | 10.9 | 2.6 | 83.8 | 2.7 | 14.0 | — | — | 100.0 | — |
| 0 | 286.3 | — | — | 1.8 | 98.2 | 237.0 | — | — | — | 100 |
| 7 | 224 | 5.2 | 7.8 | 13.4 | 73.6 | 88.7 | 2.0 | 2.3 | 6.0 | 89.7 |
| 14 | 197 | 5.8 | 9.4 | 13.7 | 71.1 | 55.3 | 3.4 | — | 7.6 | 89.0 |
| 25 | 133.7 | 7.4 | 11.5 | 20.9 | 60.2 | 33.3 | — | — | 12.3 | 87.7 |

[a] 1.5 mg BHT added per septum.
[b] (—) not detected.

(Davis et al., 1984). Table 9 presents isomerization of 8,10-dodecadienes with acetate and aldehyde functions with time. The data for 8,10–12 : Ac suggests the following equlibration:




The general pattern of isomerization of the 8,10–12 : Alds was difficult to determine as, after 25 days, the amount of aldehyde remaining in the septa was so low that detection of isomers other than the original was not always possible. Geometrical isomerization of the chemical lures may have affected captures or, indeed, may have excluded some species from field traps. Yet some lures tested in this study were quite species specific.

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# FUROCOUMARINS IN SHOOTS OF *Pituranthos triradiatus* (UMBELLIFERAE) AS PROTECTANTS AGAINST GRAZING BY HYRAX (PROCAVIIDEA : *Procavia capensis syriaca*)

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**Abstract**—*Pituranthos triradiatus* (Umbelliferae) in the Negev desert of Israel is rarely grazed and when grazing occurs, it is mostly confined to sprouts. It was hypothesized that furocoumarins accumulating in the older shoots acted as natural protectants against grazing. This was tested using hyrax (*Procavia capensis syriaca*) for bioassay. This diurnally active herbivore is known to be resistant to various poisonous plants.

Tests were conducted in the autumn (November) and summer (June). In November starved hyraxes were offered unlimited amounts of thawed, previously frozen old branches of *P. triradiatus.* They consumed an average amount of 3.4 g dry matter/kg body wt. The animals preferred to stay in the sun, and 3–4 hr after feeding, they showed severe photosensitization symptoms: apathy, photophobia, and injuries around the eyes and on the back. During the following 20 hr, four of the five treated animals died. In the second test, the effect of old branches, compared with young ones was evaluated in sunlight and under shade. Only animals that had eaten old branches and had been left in the sunlight developed photosensitization symptoms. Animals offered old shoots consumed smaller amounts than those offered young ones. However, they ingested larger amounts of imperatorin and isoimperatorin. It is suggested that these two furocoumarins induced photosensitization. Under all conditions, the hyraxes ate very small amounts of shoots of *Pituranthos*, compared with the amount of their usual diet. This suggests the presence of a severe deterrent factor, possibly furocoumarins, in the shoots. Since furocoumarins undergo light-induced cross-linking with DNA strands, it is conjectured that these natural protectants are segregated from regions within the plant where mitosis occurs, and this is why young shoots of *Pituranthos* contain smaller amounts of furocoumarins and are more susceptible to various herbivores than are old ones.



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**Key Words**—*Pituranthos triradiatus, Procavia capensis syriaca,* Procaviidae, furocoumarins, natural plant protectants, photosensitization, Umbelliferae.

## INTRODUCTION

Populations of *Pituranthos triradiatus* (Hochst ex Boiss.) Aschers and Schweinf (Umbelliferae) occur along runnels and wadi beds in various parts of the Israeli Negev desert where the annual rainfall is 50–100 mm. Shoots of this perennial shrub (1.0–1.5 m height) have small leaves that fall off during summer. Branches have terminal inflorescences that set fruits in September–October, soon after their terminal parts die. The lower parts of the old branches are green all the year round, but are grazed only to a limited extent. Information from Beduin shepherds suggests that sheep never graze on *P. triradiatus* and that whenever camels or goats browse on the plant, blood appears in their urine (Sheik Oude Shleibee, personal communication).

Recent work showed that the branches contain various furocoumarins (Figure 1) in considerable amounts (0.6–1.7% on dry weight basis) (Ashkenazy et al., 1983). When eaten by various animals (e.g., ducklings, geese, sheep, and cattle) that are subsequently kept in direct sunlight, furocoumarins have photosensitizing effects (Binns, et al., 1964; Egyed et al., 1974, 1975; Eilat et al., 1975).

Field observations showed that when browsing occurs, mammals, as well as some insects, concentrate on sprouts or on the upper parts of young shoots. Therefore, we deemed it of interest to investigate whether a diurnal indigenous herbivore, which is sympatric with a population of *P. triradiatus,* would be differently affected by browsing on young rather than on the old shoots.

## METHODS AND MATERIALS

Plant material was harvested in a natural population, near Revivim (Western Negev). A few hours after harvesting, the material was frozen at −22–24°C until thawed and fed.

The hyrax (Procaviidae: *Procavia capensis syriaca,* Figure 2) is a diurnally active herbivore. Some of its scattered populations are found where those of *P. triradiatus* occur and browze on a wide variety of plants including some poisonous plants, such as *Nerium oleander* (Meltzer, 1965) and *Phytolacca decandra* (Sale, 1965).

Animals selected for study were born in the Canadian Center for Ecological Zoology at Tel Aviv University; their parents were captured near the Dead Sea, Israel. Preliminary observations showed that satiated hyraxes avoided eating of *P. triradiutus* after one or two bites. Therefore, the effect of this plant was studied

Isopimpinellin (Isp)

Bergapten (Ber)

Isoimperatorin (Isi)

Imperatorin (Imp)

Pimpinellin

FIG. 1. Major furocoumarins in shoots of *Pituranthos triradiatus*.

on starved hyraxes. Each of 10 hyraxes was introduced into a net cage (1.1 × 1.0 × 0.8 m). In each cage a wooden box (0.50 × 0.35 × 0.35 m) was set. The box had a small entrance (0.15 × 0.14 m) and was upholstered with sawdust to stabilize inner temperature (cf. Mendelssohn, 1965). For the first 24 hr in the net cages, animals were supplied with unlimited amounts of their standard food (apples and alfalfa) and afterwards were made to fast prior to the supply of branches of *P. triradiatus*. Water was continuously supplied. Two feeding experiments were performed: one in November 1982, using only mature, old shoots of *P. triradiatus* (November harvest) and one in June 1983, using both young, new shoots, and old ones (April harvest). In the first experiment, after a 30-hr starvation period, 50 g of newly thawed shoots of *P. triradiatus* were placed in each of five of the animal cages. The other five animals were supplied again with their normal diet after fasting. The second test was aimed at comparing

FIG. 2. *Procavia capensis syriaca* (hyrax).

the effects of mature, old branches with those of young ones. The animals, after feeding, were either placed under direct sunlight or kept in the shade. Starvation period was 24 hr; control animals also fasted for 24 hr and later received their standard meal. For each treatment two animals were used.

Extraction, isolation, and chemical identification of the different furocoumarins in young and old shoots of *P. triradiatus* was performed as described by Ashkenazy et al. (1983). The isolated compounds were coinjected with standard furocoumarins for their quantitative determination in an HPLC apparatus. Quantitative determination of furocoumarins was executed separately for each harvest in November and in June.

## RESULTS AND DISCUSSION

In each of the two tests after either a 30- or 24-hr period of starvation, hyraxes consumed an average of 240–260 g/animal of standard diet, but those offered *Pituranthos* consumed only a few grams (Tables 1 and 2).

In the first test much larger amounts of dry material, as well as of furocoumarins, were consumed. This is probably due to the low temperatures which prevailed in November 1982 and the starvation period of 30 hr. An average of 9.4 ± 4.7 g of fresh *P. triradiatus* shoots per animal, or 7.5 g/kg body weight was consumed by the starved hyraxes. There was an average of 42.2 mg furocoumarins/kg body wt. in this amount of plant material. Most furocoumarins

TABLE 1. AVERAGE[a] AMOUNTS OF OLD SHOOTS OF *Pituranthos triradiatus* (HARVESTED IN NOVEMBER) AND OF FUROCOUMARINS CONSUMED BY STARVED HYRAXES (*Procavia capensis syriaca*)

| Plant material (g/kg body wt) | | Furocoumarins[b] (mg/kg body wt) | | | | | |
|---|---|---|---|---|---|---|---|
| Fresh | Dry | Isp | Ber | Imp | Cni | Isi | Total |
| 7.45 ± 2.27 | 3.39 ± 1.45 | 2.57 ± 0.75 | 2.41 ± 0.73 | 13.58 ± 4.10 | 3.02 ± 0.91 | 21.26 ± 6.34 | 42.2 ± 12.26 |

[a]Average of five animals.
[b]Isp = isopimpinellin; Ber = bergapten; Imp = imperatorin; Cni = cnidilin; Isi = isoimperatorin.

TABLE 2. AVERAGE[a] AMOUNTS OF YOUNG OR OLD SHOOTS OF *Pituranthos triradiatus* (HARVESTED IN APRIL) AND FUROCOUMARINS CONSUMED BY STARVED HYRAXES (*Procavia syriaca capensis*)

| | | Plant material (g/kg body wt) | | Furocoumarins[b] (mg/kg body wt) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Branch age | Fresh | Dry | Isp | Ber | Imp | Cni | Isi | Total |
| Sunlight | Young sprouts | 5.83 ± 0.07 | 2.06 ± 0.19 | 1.64 ± 0.01 | 1.23 ± 0.02 | 1.62 ± 0.03 | 0.62 ± 0.02 | 3.09 ± 0.03 | 8.21 ± 0.17 |
| | Old | 1.96 ± 0.18 | 0.79 ± 0.06 | 0.66 ± 0.05 | 0.61 ± 0.07 | 3.48 ± 0.33 | 0.52 ± 0.32 | 5.41 ± 0.52 | 10.68 ± 0.42 |
| Shade | Old | 2.65 ± 0.62 | 1.06 ± 0.24 | 0.88 ± 0.33 | 0.78 ± 0.16 | 4.48 ± 0.29 | 0.98 ± 0.49 | 6.82 ± 0.19 | 13.72 ± 0.58 |

[a]Average of two animals.
[b]Isp = isopimpinellin; Ber = bergapten; Imp = imperatorin; Cni = cnidilin; Isi = isoimperatorin.

consisted of isoimperatorin and imperatorin (21.2 mg/kg body wt. of isoimperatorin and 13.6 mg/kg body wt. of imperatorin). Other furocoumarins were cnidilin, bergapten, and isopimpinellin, each of which was consumed in amounts of 2.4–3.0 mg/kg body wt. (Table 1). Three to four hours after eating *Pituranthos,* all five treated animals exhibited syndromes of photosensitization, e.g., apathy, photophobia, loss of hair in tiny spots, followed by small injuries on the back and particularly around the eyes (Figure 3). After 24 hr, four hyraxes died. No such effects could be seen in the control fasting animals. Postmortem examinations showed the presence of undigested material of branches of *Pituranthos* along the gastric tract, ulcers scattered in the fundic region, and swollen adrenal glands with acute hemorrhages. Since not all the shoots were digested, it was assumed that only a fraction of the furocoumarins within the consumed plant entered the blood system of the animal. Acute toxic effects followed by death of the animals were, at least in part, due to an interaction with the low autumn temperatures prevailing in Tel Aviv during the time of the first experiment. These temperatures, which forced the animals to stay in the sun after ingestion of *Pituranthos,* could also reduce their resistance.

FIG. 3. Injuries around the lower part of the eye of a hyrax that appeared 3–4 hr after ingestion of old branches of *Pituranthos triradiatus,* in animals kept under sunlight.

The second test was performed in the early summer (June). Here, animals consumed only 1.9 g/kg body wt. of old branches in June, as compared with 3.4 g/kg body wt. in November. At this time of the year the animals tended to enter their boxes as a shelter against the sun. Therefore, in each treatment, two animals were allowed to use their boxes and their cages were shaded, whereas two other animals were left exposed to the sun soon after eating for a 3-hr period. Only animals fed with old branches that were kept in sunlight exhibited syndromes of photosensitization. The hyraxes in shaded cages consumed almost 35% more old branches of *Pituranthos* than those kept in sunlight. Thus, despite ingestin of more furocoumarins by the animals kept in the shade (Table 2), they did not show the syndrome of a photosensitization response.

Only the combination of sunlight and furocoumarins resulted in photosensitization. Photosensitized animals consumed somewhat less of the plant than did those showing no photosensitization effect. Animals under sunlight, fed young branches, consumed three times more fresh plant material than did those fed old branches. Since the concentration of furocoumarins in old branches is higher than in the young ones, the total amount of furocoumarins introduced into the hyraxes that ate old branches was higher than in those that consumed young ones (Table 2). This is probably why photosensitization appeared only in those animals that ate old branches. It has already been proposed that furocoumarins function as deterrents against insects (Berenbaum, 1978) and mammals (Ivie, 1978). Both tests support this. When animals were offered young or old branches the young branches were consumed 2.5 times more eagerly than the old ones, but both groups of animals consumed similar amounts of furocoumarins (Table 2). The conspicuous photosensitization and the possible repellent effects of the furocoumarins on the hyraxes seem to act as an efficient protective system of *Pituranthos triradiatus* against an herbivore which is usually resistant to various poisonous plants. However, wild animals might be more resistant.

By eating old shoots, 2.1 and 1.7 times more imperatorin and isoimperatorin, respectively, were consumed than had young shoots been eaten. The others, isopimpinellin, bergapten and cnidilin, were ingested in smaller amounts than by eating the young shoots. It is therefore possible that imperatorin and/or isoimperatorin in shoots of *P. triradiatus* are the major photosensitizers.

Examination of furocoumarins in old and young shoots of *P. triradiatus* in five of six populations showed a pattern of distribution similar to that found in the Revivim population (Figure 4). The Zin population deviates from the rest. Imperatorim was found to be much reduced and toxicity may be preserved by the higher concentration of bergapten. Compared with the first test (November), no death occurred in June. In the second test, a shorter period of starvation could reduce the amounts of consumed plant material and a four times smaller amount of furocoumarins was thus ingested by the hyraxes (cf. Tables 1 and 2).

The considerably lower amounts of furocoumarins in the young shoots as compared with old ones was found in plants from all six different localities

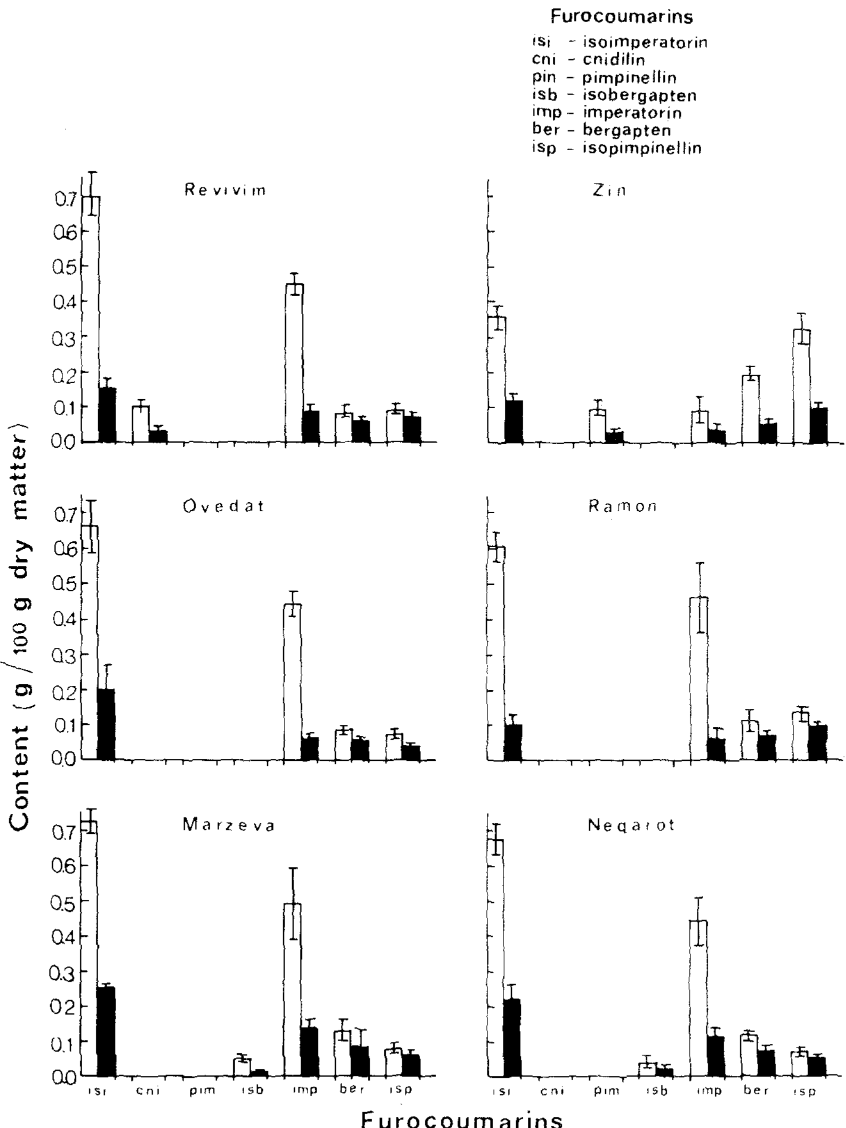


FIG. 4. Content of furocoumarins (g/100 g dry matter, %) in old (open columns) and young shoots (dark columns) in six different populations of *Pituranthos triradiatus*, along a transect of 80 km.

(Figure 4). This explains the relative susceptibility of young shoots to various mammalian herbivores.

Although in general, young shoots, compared with the old ones, are rich in concentrations of secondary metabolites (cf. McKey, 1979), in young *Pituranthos*

branches, concentrations of furocoumarins per unit dry weight are lower than in older ones. Since these compounds interfere with DNA replication (Scott et al., 1976) and since mitosis in young branches is common, the low concentrations of these compounds are selected to avoid autotoxic hazards (cf. Friedman and Waller, 1984). Plants that store furocoumarins as protection against grazing may not be able to protect their young branches during growth, but may use them as an efficient means to protect mature plant organs against diurnal herbivores.

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# POSSIBLE PHEROMONAL REGULATION OF REPRODUCTION IN WILD CARNIVORES

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**Abstract**—Recent observations of social behavior and reproduction in wolves, coyotes, hunting dogs, mongooses, and lions suggest possible involvement of pheromones in the regulation of reproductive activity. Observed phenomena resemble the known pheromonal effects in mice such as suppression or synchronization of estrus, induction of maternal behavior, and interruption of pregnancy. Further studies are necessary to verify the supposed biological effects of carnivore pheromones and to determine their chemical nature.

**Key Words**—Wolf, coyote, hunting dog, mongoose, lion, estrous synchrony, estrous suppression, pregnancy interruption, sexual pheromones, scent marking.

## INTRODUCTION

Pheromonal influences on reproduction of laboratory rodents are well known (Bronson, 1974). These effects apparently exist in a variety of other mammal species including man, but their study is more difficult than in laboratory animals. Nevertheless, there are reports of animal behavior which suggest probable involvement of pheromones. Reevaluation of these results from the point of view of contemporary knowledge of the pheromonal physiology may explain some patterns of social behavior and reproduction in carnivores.

Recent zoological literature contains this kind of information on several carnivore species. This paper deals with the possible pheromonal regulation of reproduction in group living animals such as wolves (*Canis lupus*), coyotes (*Canis latrans*), hunting dogs (*Lycaon pictus*), bush dogs (*Speothos venaticus*), bat-eared foxes (*Otocyon megalotis*), yellow mongooses (*Cynictis penicillata*),



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banded mongooses (*Mungos mungo*), dwarf mongooses (*Helogale undulata* and *H. parvula*), lions (*Panthera leo*), and pumas (*Felis concolor*).

## GROUP-LIVING CANIDS

Wild wolves, coyotes, or hunting dogs live in groups (packs) consisting of a dominant leading (alpha) pair and its subordinate offspring of different ages. Scent marking with urine in these group-living canids is related to their social hierarchy and reproductive activity. Most of the marking is done by the dominant pair which marks all territorial landmarks and also the caches and food eaten by other members of the pack. Subordinate animals contribute only a little to the territorial scent marking. Scent marks are investigated by all members of the group and certainly contain olfactory signals asserting the social dominance of the leading pair. A sharp increase in urine marking of the empty food caches by both dominant and subordinate animals after the food has been consumed has been described as a "bookkeeping" behavior signaling the other animals that the cache is empty and not worthy of further checking (Blizard and Perry, 1979; Harrington, 1981, 1982; Henry, 1977, 1980). However, it may instead be triggered by the decomposition odors which are potent inducers of marking behavior and which were masked before by odors of edible food.

Scent marking in the alpha pair intensifies during breeding season, which suggests its close relation to reproduction. The pair shows an interesting phenomenon of "double marking" with urine. Each spot where the dominant female has urinated is thoroughly sniffed by the dominant male and then overmarked with his urine. This behavioral pattern is sometimes explained as a means of hiding the female's reproductive status from other males, but its exact biological significance is not known (Barrette and Messier, 1980; Bekoff and Diamond, 1976; Frame et al., 1979; Frame and Frame, 1976; Peters and Mech, 1975; Rothman and Mech, 1979; Van Heerden, 1981).

Within the wild pack only the dominant pair is regularly reproductively active and only the alpha female gives birth and raises young. Subordinate animals usually do not reproduce and the rare young born to a subordinate female have a poor survival rate (Harrington et al., 1982; Peters and Mech, 1975; Rothman and Mech, 1979; Zimen, 1981). The dominant female also exhibits estrous hormonal changes while in the subordinate females these changes are absent or irregular (Seal et al., 1979). Under rather artificial and crowded conditions of captivity, a few subordinate female wolves showed some sexual activity, but they never gave birth to young. The dominant pair of this captive wolf pack regularly produced one litter per year (Woolpy, 1968).

Occasionally individual subordinate animals leave the suppressing environ-

ment of the pack and wander over a large territory until they find pairmates and vacant territories where they can form new breeding pairs. The new pairs show very intensive territorial scent marking and double marking. While the territorial marking certainly demarcates the newly occupied territory, the double marking may be related to reproduction of the pair (Peters and Mech, 1975; Rothman and Mech, 1979).

Overall suppression of the marking and reproductive activity in subordinate animals of the pack certainly influences their endocrine functions. This suppression may be mediated by chemical signals emanating from scent marks of the frequently marking leading pair. Deposition of its scent marks to the frequently investigated places (caches) and even to the food may ensure effective suppression. Suppressing cues may be identical to chemical signals asserting the social hierarchy, since the social dominance, scent marking, and reproductive activity are hormonally dependent (Berg, 1944; Johnson, 1973; Peters and Mech, 1975; Ralls, 1971; Rothman and Mech, 1979). The dominant pair that induces reproductive suppression is apparently uninfluenced by its own scent marks. This inertness of animals to their own pheromones is known also in mice (Bronson, 1974). Similar social suppression of the reproductive functions in subordinate females has been observed in groups of primates (Abbott and Hearn, 1978; Abbott et al., 1981).

Reproductive activity and breeding synchrony may be induced by a specific priming chemical signal. Winter reproductive activity in males starts earlier than in females but ends nearly simultaneously in both sexes. Also the reproductive activity of the new breeding pair starts with some delay after the pair formation (Bekoff and Diamond, 1976; Peters and Mech, 1975; Rothman and Mech, 1979). The existence of chemical communication of reproductive status within the breeding pair can be inferred from an observation of caged red foxes. Application of estradiol benzoate to a vixen increased the urinary levels of reproduction-related 3-methylbutyl methylsulfide in both the female and a male in the next cage (Bailey et al., 1980). It is possible that this sulfur compound itself or some other induced airborne chemical signal was responsible for this phenomenon.

Double marking may lead to the formation of the priming signal. Male dog urine masks the attractivity of female estrous urine (Dunbar and Buehler, 1980) despite the keen sense of smell in carnivores. Contact of the male and female urine may trigger a biochemical reaction rather than simply mask the odor of estrus-related compounds. Compounds of the male and female urine may then form a priming product which occurs after both animals have reached a certain stage of reproductive readiness. This product then may trigger the reproductive activity of the pair and thereafter regulate it synchronously. Frequent mutual olfactory investigation of scent marks would ensure sufficient exposure to the responsible chemical signals from the pairmate.

The double marking pattern has been observed also in bush dogs (Porton,

1983), bat-eared foxes (Lamprecht, 1979; Nel and Bester, 1983) and other wild canids (Macdonald, 1979, 1980), and it has been noted also in zebras (*Equus burchelli boehmi*) (Klingel, 1967, 1969).

Reproductive suppression within the packs has a feature of natural population control. Full use of all territories by existing packs prevents formation of new pairs and, therefore, only established dominant breeding pairs generate young which are, however, suppressed in further reproduction. Death from disease or shooting destroys some packs, and their vacant territories are quickly occupied by new breeding pairs recruited from subordinates of the existing packs. Average size and age structure of the packs and the size of the pack territory may be an indicator of population turnover. A higher proportion of pairs and smaller packs would mean a higher percentage of the population actively engaged in reproduction and vice versa. Disruption of the supposed olfactory communication within the breeding pair by alteration or suppression of sexual odor production might be a way of controlling the population of predaceous carnivores (Bekoff and Diamond, 1976).

## MONGOOSES

Yellow, banded, and dwarf mongooses live in family groups with a structure similar to that of wolf packs. Most of the scent marking is done by the dominant pair, while the subordinates contribute only a little (Earlé, 1981; Rasa, 1977; Rood, 1975, 1980).

Adult females in the group of banded mongooses usually show very synchronous estrous cycles and births. Reproductive synchrony is induced by social factors rather than physical environment, since neighboring groups of mongooses have their own synchrony independent of each other (Rood, 1975).

Dwarf mongooses also show synchronous estrus but only the dominant female regularly bears young. Subordinate females may not conceive or may abort early since they show no signs of pregnancy. However, the subordinate females often start lactation after parturition in the dominant female; this may be important in the communal nursing system in mongooses. Sexual maturation of young females is delayed in the suppressing environment of the group. Adult subordinate females quickly resume full reproductive activity after leaving the parental pack and becoming dominant females in new pairs or established packs (Rood, 1980).

It is interesting that, under space restriction of captive groups of dwarf mongooses, all adult females show clear estrous synchrony and give birth but the young usually do not survive. When more space is allowed for the group, reproduction is again suppressed in the subordinate females, and they show no more pregnancies (Rasa, 1973, 1979).

## LIONS

Lions in the wild live in social units (prides) consisting usually of two to four adult males, five to nine adult females, and dependent cubs. Average behavioral estrus lasts about four days, interestrus 16 days, and pregnancy 110 days. The average number of young born is two to three. Lionesses with dependent young do not return to cyclic sexual activity until the cubs are 1.5–2 years old but quickly return to it once they have lost their dependent offspring. Litters of cubs born nearly synchronously have a much better survival rate than cubs from small litters born asynchronously. Low survival of these young is caused partly by insufficient nutrition since the female allows suckling by older cubs. Lion prides show marked estrous and birth synchrony which is little dependent on environmental factors, since it has been observed in two neighboring prides independently of each other, and lions generally do not have any seasonality in reproduction (Bertram, 1975; Packer and Pusey, 1983a,b; Rudnai, 1973; Schaller, 1972).

From time to time there is a male takeover in the leadership of a lion pride which means installation of a new group of breeding males. Shortly after the takeover, most of the dependent young up to 2 years old are killed, die by emaciation, or are expelled from the pride and eventually die. Most females which have lost their young return to synchronous estrus within a few days after the litter loss. Very few births have been observed during the 7-month period after takeover and most of them occurred within the first two months. Nevertheless, the young rapidly disappeared, and the females resumed synchronous estrous cyclicity. Despite frequent copulations with the new males, the females do not conceive earlier than 4–5 months after takeover. Estrous synchrony is followed by less sharp conception and birth synchrony (Bertram, 1975; Packer and Pusey, 1983a,b; Rudnai, 1973; Smuts et al., 1978).

These reproductive phenomena in lions may have a pheromonal background. Male takeover resembles the introduction of a strange male to the mouse female at early pregnancy when the pregnancy is interrupted and incidental new conception with the new male takes place (Bruce, 1960). A similar effect has been observed in wild horses where the male takeover of a herd caused abortions of up to 6-month-old pregnancies (about one half of gravidity) and conceptions with the new stallion (Berger, 1983). Apparently the phenomenon of pheromonal pregnancy interruption is not rare and may have different time and other parameters in various species.

It is possible that the new male lions pheromonally interrupted all pregnancies up to one half of the gravidity length (Bertram, 1975; Packer and Pusey, 1983a,b). Longer pregnancies resulted in births within one to two months after the male takeover with no young surviving. This would indicate that the pregnancy in its second half may resist male influence but the onset of lactation and maternal care is profoundly influenced. Hormonal changes simultaneously in-

duced by the male influences on the females during the first half of pregnancy could produce a uterine environment incompatible with embryo survival and return the females to cycling activity. Similar hormonal changes in the nursing lionesses could interfere with lactation and maternal care and also return the females to cycling activity. Hormonal changes in females could include suppression of prolactin levels since this hormone has been found to both prevent pheromonal pregnancy interruption in mice and support the lactation and maternal behavior in parturient females (Chapman et al., 1970; Dominic, 1967; Poindron and Le Neindre, 1980). Lack of maternal care and milk together with direct killing could be the main reason for the high cub mortality after the takeover.

Male-induced hormonal changes might prevent ovulation, conception, and/or implantation in females for several months despite regular and synchronous behavioral estrus and frequent multiple copulations (Eaton, 1974, 1978; Smuts et al., 1978). Behavioral estrus signs often are not accompanied by corresponding hormonal changes (Eaton, 1974; Schmidt et al., 1979). This infertile period lasts six to nine cycles, and its length in individual females depends on the time of abortion or litter loss after the takeover. Persistence of synchronous estrous cyclicity could be maintained by a communal surge of ovarian activity through priming chemical cues originating from females (Eaton, 1974; Smuts et al., 1978). Similar estrous synchronization has been already observed in a cat colony (Bland, 1979; Hay and Graham-Marr in Bland, 1979). Examinations of ovaries in a lion pride shot during synchronous estrus have shown high follicular activity in all females. The finding of a 1-cm follicle in a lioness determined as three weeks pregnant indicates the power of social stimuli to induce ovarian and/or behavioral estrous changes despite the beginning pregnancy (Smuts et al., 1978). The occurrence of pseudoestrus during pregnancy in lions is not rare (Packer and Pusey, 1983a).

Expiration of the male block of implantation or accommodation of male–female reproductive relationships together with synchronous estrous activity may lead to synchronous conception in the majority of females within a time span of two consecutive cycles (three periods of estrus). On average, the females have been observed to conceive 134 (range 100–164) days after male takeover, while females which have lost young for other reasons conceived, on average, after 24 days (Packer and Pusey, 1983a,b). Nearly synchronous conception consequently results in nearly synchronous births. The collective effect of pheromonal influences associated with synchronized parturitions and young may induce the optimal environment for survival and raising of young in the pride communal system. It is possible that a female nursing few young born asynchronously does not have pheromonal stimulation strong enough to maintain lactation and maternal care until weaning. Importance of the power of social environment tuned by synchronous births for the communal raising of the young can be inferred from observations that male takeover may fail to induce the described reproduc-

tive changes if the majority of pride females nurse very synchronous litters of small cubs and cooperatively protect them (Packer and Pusey 1983a,b).

Male takeovers and resulting reproductive synchronization in the wild lion prides certainly have evolutionary advantages in maintaining genetic heterogeneity and in better survival of young. Sharp synchronization induced after male takeover becomes diffuse in time, but it is probably frequently renewed. Assuming an average pregnancy length of 110 days and 1.5–2 years of nursing anestrus, the female can successfully raise a litter every other year. Male tenure within the pride is about one to four years, depending on the number of males (Bertram, 1975; Packer and Pusey, 1983a), which means that probably every or every other birth period follows a male takeover with updated synchronization. High frequency and inefficiency of mating may elicit increased intermale aggression and ensure that larger coalitions of leading males will take over the pride and keep it for a longer time than a small group of males. This would give a better chance for complete raising of a generation of young (Packer and Pusey, 1983a).

Prides of captive lions subjected to more detailed observations also show estrous synchrony. It is induced by a female coming into true physiological estrus or approaching parturition. Her urine is very attractive for males which indicates a probable role of urinary pheromones in regulation of reproductive functions. The female in true estrus is more than twice as likely to conceive as the other females with induced estrus. Apparently, in many of these females only behavioral signs of estrus without corresponding hormonal changes and ovulation are induced (Eaton, 1974).

Most of the captive pride members participate in reproduction but the dominant animals tend to be more active. There is some evidence that the dominant male sires most of the cubs, although all males participate in mating. Conception rate in females is less dependent on their social rank, but subordinate females show weaker maternal behavior. Their young are often abandoned or adopted by other females. However, most prides have some low-rank females that do not reproduce at all. It is not clear if there is a reproductive suppression similar to that in wolves or a pathological reproductive disorder (Eaton, 1974).

Captive pumas (*Felis concolor*) kept in a group have also showed certain estrous synchrony, although these animals in the wild are rather solitary. The synchrony is induced by a female coming into true physiological estrus with mating. Other females probably show only behavioral estrus, since they do not always mate with males (Eaton and Velander, 1977).

## CONCLUSION

Examples presented of reproductive phenomena in carnivores have some common features with pheromonal influences on reproduction in laboratory ro-

dents. Further studies should verify the supposed biological effects of pheromones in carnivores and search for the chemical signals involved.

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# A MULTICHEMICAL DEFENSE MECHANISM OF BITTER OLIVE *Olea europaea* (OLEACEAE) Is Oleuropein a Phytoalexin Precursor?

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**Abstract**—*Olea europaea* (Oleaceae) is resistant in nature to insect and microbe attack. Two types of chemical protection were found in the foliage. One type is the bitter *seco*-iridoid glycosides oleuropein (1) and ligstroside (2); The other is a physical barrier of crystalline oleanolic acid (4) that coats the leaf surface. The *seco*-iridoid glycosides were isolated using two different countercurrent chromatographies: rotation locular countercurrent chromatography (RLCC) and droplet countercurrent chromatography (DCCC). The dimethyl ester (III) was shown to be an artifact. This is the first isolation of ligstroside from *O. europaea*. In an antimicrobial test by the paper disk method against *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Escherichia coli*, compounds I, II, and III inhibited a growth of *B. subtilis* at pH 7. Similar tests under the influence of $\beta$-glucosidase suggest an aglycone of oleuropein, either the hemiacetal (i) or the possible enal-aldehyde (ii), could be the active intermediate. This intermediate could be produced rapidly in response to microorganism invasion. Oleuropein producing such a postinfection active intermediate could be referred to as a phytoalexin precursor.

**Key Words**—*Olea europaea* (Oleaceae), oleuropein, ligstroside, oleanolic acid, rotation locular countercurrent chromatography (RLCC), droplet countercurrent chromatography (DCCC), chemical barrier, multichemical defense mechanism, enal–aldehyde active intermediate, phytoalexin precursor.

## INTRODUCTION

Host-plant resistance to attack by insect and microbe is mediated by chemical agents. An investigation of these agents is an important part in understanding evolutionary and ecological aspects of plant-microbe relationships, and may also



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have some practical use. We have investigated a number of plants that are resistant to insect and microbe attack in order to identify the agents responsible for this resistance (Kubo et al., 1984). Recently our investigation has centered on the bitter olive, *O. europaea* (Oleaceae).

This paper describes the isolation and characterization of two classes of chemicals from *O. europaea* that contribute to its resistance to microbe attack and proposes the structural moiety responsible for the observed antimicrobial activity.

*O. europaea* is known to be relatively immune to microbe and insect attack. At least some of this immunity may be attributed to a high concentration of *seco*-iridoid glycosides (I and II), which occur predominantly in the Oleaceae family (Asaka et al., 1972). The resistance can also be attributed to a large amount of oleanolic acid (IV) on the foliage that acts as a physical barrier. Although oleanolic acid itself does not exhibit antimicrobial activity, it covers almost all of the leaf. This is undoubtedly important in limiting the penetration of microbe into the plant tissue since the microbe cannot germinate and grow unless sufficient moisture is available within the leaf phillosphore.

## METHODS AND MATERIALS

*Materials.* Fresh fruits of *O. europaea* (2.1 kg) were collected on the UC Berkeley campus. After extraction for more than six months with methanol, the methanol was removed and 162.4 g of extract was obtained.

*DCCC Separation for I.* The DCCC separation was performed on a model DCCC-300-G2 (Tokyo Rikakikai Co., Tokyo, Japan). The DCCC solvent system of $CHCl_3$–$CH_3OH$–$H_2O$ (13 : 7 : 4, v/v) was chosen by prescreening the main compounds of an initial methanol extract on a TLC plate (Macherey, Nagel, and Co., Duren, G.F.R., poligram Sil G/UV 254). The upper phase was chosen as the mobile phase in our DCCC system. The crude methanol extract (1.0 g) was dissolved in a (1 : 1, v/v) mixture of the mobile and stationary phases and injected into the DCCC apparatus using a 10-ml sample coil. The eluate was collected in 2.2-ml fractions. Fractions were monitored by TLC (Sil G/UV 254) developed with the organic layer of this solvent system. Visualization was accomplished by UV (Chromato-UVE Cabinet, model CC-60, Ultra violet products, Inc., California) and a vanillin–sulfuric acid–ethanol (3 g : 1.5 ml : 100 ml) spray reagent. The DCCC chromatogram is shown in Figure 1.

*DCCC Separation for II and III.* The mixture sample (87 mg), collected in fractions 49–72 (111 mg) of the DCCC separation for I, was reinjected using $C_6H_6$–$CHCl_3$–$CH_3OH$–$H_2O$ (5 : 5 : 7 : 2, v/v) solvent system in the ascending method. The eluents were collected in 2.2 ml fraction. The DCCC chromatogram is shown in Figure 2.

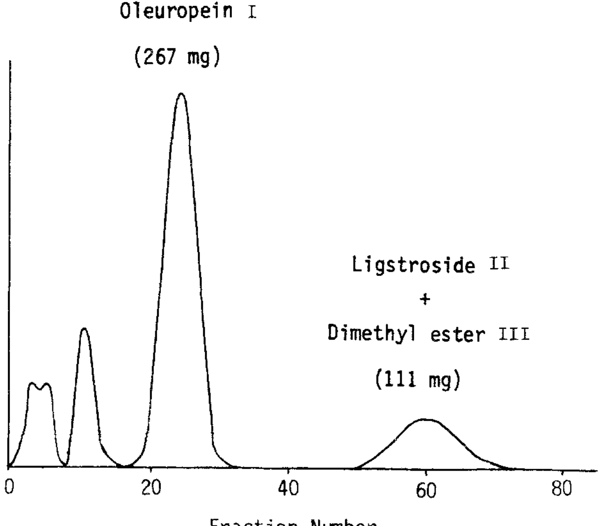


FIG. 1. DCCC of the methanol extract of *O. europeae* (1.0 g) with $CHCl_3$–$CH_3OH$–$H_2O$ (13:7:4, v/v) by the ascending method; 2.2 ml/fraction; 5 days.

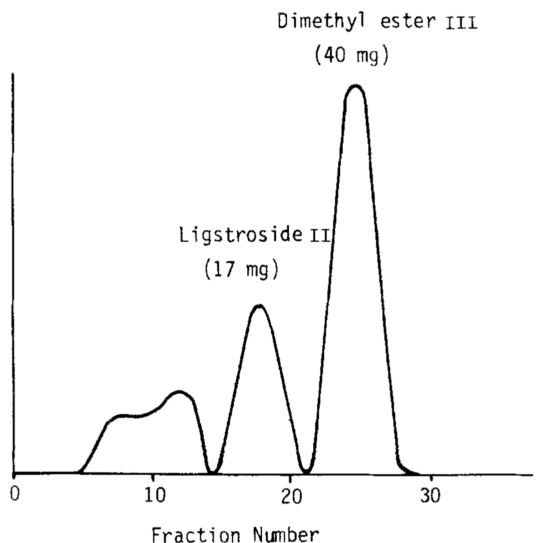


FIG. 2. DCCC of the mixture (87 mg) of IV and V with $C_6H_6$–$CHCl_3$–$CH_3OH$–$H_2O$ (5:5:7:2, v/v) by the ascending method; 2.2 ml per fraction.

*Oleuropein (I).* Amorphous (Scheme 1). $[\alpha]_D^{28}$ −146.2° (c = 0.44, MeOH). UV (EtOH) 228.5 nm (ε = 18000), 278.0 nm (ε = 3300). IR (Nujol) 3100–3500 (br OH), 1680–1725 (br COO), 1620, 1520 (arom.) $cm^{-1}$. [$^1$H]NMR (DMSO-$d_6$) δ 1.65 (3H, d, $J$ = 6 Hz, 10-H), 2.41 (1H, dd, $J$ = 14 Hz, 9 Hz, 7-$H_a$), 2.63 (1H, dd, $J$ = 14 Hz, 4 Hz, 7-$H_b$), 2.69 (2H, t $J$ = 6 Hz, 7′-H), 3.65 (3H, s, 12-H), 3.86 (1H, dd, $J$ = 9 Hz, 4 Hz, 4-H), 3.95–4.18 (2H, m, 8′-H), 4.66 (1H, d, $J$ = 8 Hz, anomeric-H), 5.87 (1H, s, 2-H), 5.97 (1H, q, $J$ =6 Hz, 9-H), 6.48 (1H, dd, $J$ = 8 Hz, 2 Hz, 6′-H), 6.61 (1H, d, $J$ = 2 Hz, 2′-H), 6.65 (1H, d, $J$ = 8 Hz, 5′-H), 7.53 (1H, s, 6-H), 8.73 (1H, d, $J$ = 8 Hz, OH). [$^{13}$C]NMR (DMSO-$d_6$) δ 12.9 (q), 30.1 (d), 33.7 (t), 39.9 (t), 51.2 (q), 61.1 (t), 65.0 (t), 69.9 (d), 73.3 (d), 76.5 (d), 77.3 (d), 92.9 (d), 99.0 (d), 107.7 (s), 115.5 (d), 116.1 (d), 119.5 (d), 123.0 (d), 128.4 (s), 129.1 (s), 143.7 (s), 145.0 (s), 153.4 (d), 166.2 (s), 170.0 (s). SI-MS $m/z$: 541 ($M^+$1), 427, 423, 361, 225, 137.

*Hexaacetyloleuropein (Ia).* Acetylation of I with acetic anhydride and pyridine gave Ia (Scheme 1). Amorphous. $[\alpha]_D^{25}$ −109.7° (c = 0.06, $CHCl_3$). UV (EtOH) 224.0 nm (ε = 12000). IR ($CHCl_3$) no OH, 3010, 735 (arom.), 1746, 1210 (COO) $cm^{-1}$. [$^1$H]NMR ($CDCl_3$) δ 1.70 (3H, d, $J$ = 8 Hz, 10-H), 2.05, 2.31 (12H, 6H each, both s, OAc × 6), 2.43 (1H, dd, $J$ = 14 Hz, 8 Hz, 7-$H_a$), 2.77 (1H, dd, $J$ = 14 Hz, 4 Hz, 7-$H_b$), 2.81 (2H, t, $J$ = 7 Hz, 7′-H), 3.74 (3H, s, 12-H), 3.96 (1H, dd, $J$ = 8 Hz, 4 Hz, 4-H), 5.70 (1H, s, 2-H), 6.00 (1H, q, $J$ = 8 Hz, 9-H), 7.05-7.10 (3H, m, 2′-, 5′-, 6′-H), 7.45 (1H, s, 6-H). [$^{13}$C]NMR ($CDCl_3$) δ 13.5 (q), 20.6 (q, C × 6), 30.3 (d), 34.4 (t), 40.0 (t), 51.4 (q), 61.8 (t), 64.5 (t), 68.4 (d), 70.8 (d), 72.3 (d), 72.6 (d), 93.8 (d), 97.2 (d),

| | | |
|---|---|---|
| I : | $R^1$ = OH | $R^2$ = H |
| Ia : | $R^1$ = OAc | $R^2$ = Ac |
| II : | $R^1$ = H | $R^2$ = H |
| IIa : | $R^1$ = H | $R^2$ = Ac |

SCHEME 1.

108.8 (s), 123.4 (d), 123.8 (d), 124.9 (d), 127.0 (d), 128.2 (s), 136.6 (s), 140.8 (s), 142.0 (s), 153.1 (d), 166.8 (s), 168.1 (s), 168.2 (s), 169.3 (s), 169.4 (s), 170.1 (s), 170.5 (s), 171.0 (s).

*Ligstroside (II).* Amorphous (Scheme 1). $[\alpha]_D^{28}$ −131.4° (c = 0.30, MeOH). UV (EtOH) 223.0 nm ($\epsilon$ = 18000), 273.0 nm ($\epsilon$ = 1600). IR (Nujol) 3200–3500 (br OH,), 1685–1730 (br COO), 1625, 1518 (arom.) $cm^{-1}$. $[^1H]$NMR ($DMSO-d_6$) $\delta$ 1.61 (3H, dd, $J$ = 8 Hz, 2 Hz, 10-H), 2.40 (1H, dd, $J$ = 14 Hz, 10 Hz, 7-$H_a$), 2.63 (1H, dd, $J$ = 14 Hz, 4 Hz, 7-$H_b$), 2.74 (2H, t, $J$ = 8 Hz, 7′-H), 3.64 (3H, s, 12-H), 3.84 (1H, dd, $J$ = 10 Hz, 4 Hz, 4-H), 4.05 (1H, dd, $J$ = 12 Hz, 8 Hz, 8′-$H_a$), 4.15 (1H, dd, $J$ = 12 Hz, 8 Hz, 8′-$H_b$), 4.65 (1H, d, $J$ = 8 Hz, anomeric-H), 5.86 (1H, s, 2-H), 5.96 (1H, q, $J$ = 8 Hz, 9-H), 6.68 (2H, d, $J$ = 8 Hz, 2′-, 6′-H), 7.02 (2H, d, $J$ = 8 Hz, 3′-, 5′-H), 7.52 (1H, s, 6-H), 9.24 (1H, s, OH). $[^{13}C]$NMR ($DMSO-d_6$) $\delta$ 12.8 (q), 30.1 (d), 33.4 (t), 39.8 (t), 51.1 (q), 61.1 (t), 64.9 (t), 69.9 (d), 73.2 (d), 76.5 (d), 77.3 (d), 92.9 (d), 99.0 (d), 107.6 (s), 115.1 (d, C × 2), 122.9 (d), 127.7 (s), 129.1 (s), 129.7 (d, C × 2), 153.3 (d), 155.8 (s), 166.1 (s), 170.5 (s). SI-MS $m/z$: 525 ($M^+1$), 427, 363, 331, 233, 225, 207, 121.

*Pentaacetylligstroside (IIa).* Acetylation of II with acetic anhydride and pyridine gave IIa. (Scheme 1). Amorphous. $[\alpha]_D^{25}$ −104.4° (c = 0.16, $CHCl_3$). UV (EtOH) 212.0 nm ($\epsilon$ = 16000), 223.0 nm ($\epsilon$ = 16500). IR ($CHCl_3$) no OH, 3010, 1615, 720 (arom.), 1747, 1210 (COO) $cm^{-1}$. $[^1H]$NMR ($CDCl_3$) $\delta$ 1.69 (3H, d, $J$ = 8 Hz, 10-H), 2.00, 2.30 (12H, 3H each, both s, OAc × 5), 2.40 (1H, dd, $J$ = 14 Hz 10 Hz, 7-$H_a$), 2.73 (1H, dd, $J$ = 14 Hz, 6 Hz, 7-$H_b$), 2.89 (2H, t, $J$ = 6 Hz, 2′-H), 3.71 (3H, s, 12-H), 3.96 (1H, dd, $J$ = 10 Hz, 6 Hz, 4-H), 4.05-4.37 (2H, m, 8′-$H_{ab}$), 5.66 (1H, s, 2-H), 5.96 (1H, q, $J$ = 8 Hz, 9-H), 6.96 (2H, d, $J$ = 10 Hz, 2′-, 6′-H), 7.16 (2H, d, $J$ = 10 Hz, 3′-, 5′-H), 7.39 (1H, s, 6-H). $[^{13}C]$NMR ($DMSO-d_6$) $\delta$ 13.5 (q), 20.6 (q, C × 4), 21.1 (q), 30.2 (d), 34.4 (t), 39.9 (t), 51.4 (q), 61.7 (t), 64.9 (t), 68.2 (d),70.7 (d), 72.2 (d), 72.5 (d), 93.7 (d), 97.0 (d), 108.8 (s), 121.6 (d, C × 2), 124.8 (d), 128.0 (s), 129.8 (d, C × 2), 135.2 (s), 149.3 (s), 153.0 (d), 166.7 (s), 169.2 (s), 169.3 (s), 169.4 (s), 170.1 (s), 170.5 (s), 171.0 (s).

*Dimethyl Ester (III).* Amorphous. UV (EtOH) 233.0 nm ($\epsilon$ = 11000). IR (Nujol) 3520–3200 (br OH), 1730, 1700 (br COO), 1630 (br C = CH), 1010–1090 (br COC) $cm^{-1}$. $[^1H]$NMR ($DMSO-d_6$) $\delta$ 1.68 (3H, d, $J$ = 7 Hz), 2.43 (1H, dd, $J$ = 15 Hz, 10 Hz). 2.66 (1H, dd, $J$ = 15 Hz, 4.5 Hz), 3.07 (1H, m), 3.04–3.13 (1H, m), 3.16–3.26 (2H, m), 3.46 (1H,m), 3.57 (3H, s), 3.65 (3H, s), 3.69 (1H, m), 3.86 (1H, dd, $J$ = 10 Hz, 4.5 Hz), 4.47 (1H, t, $J$ = 7 Hz), 4.65 (1H, d, $J$ = 8 Hz), 4.98, 5.04 (both 1H, both br s), 5.17 (1H, d, $J$ = 4 Hz), 5.85 (1H, s), 5.97 (1H, q, $J$ = 7 Hz), 7.53 (1H, s), Assignments shown in Figure 3 are confirmed by decoupling experiments. $[^{13}C]$NMR ($C_5D_5N$) $\delta$ shown in Figure 3. EI-MS $m/z$: 256, 238, 196, 178, 165, 151.

*Tetraacetyldimethyl Ester (IIIa).* IIIa was obtained from III by the same way as for Ia. Physical and spectral data of IIIa agreed well with those of the authentic

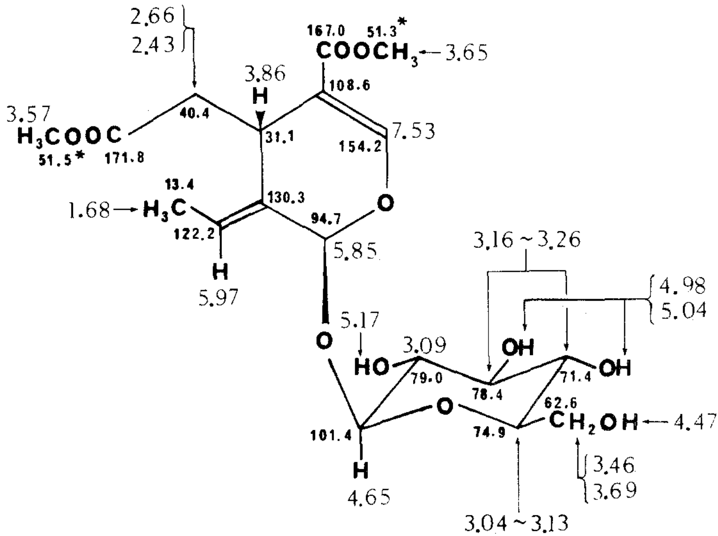


FIG. 3. ^{1}H NMR and ^{13}C NMR assignments for III. *Assignments may have to be interchanged. Decoupled experiments also confirmed the ^{1}H NMR assignments.

sample (Inoue et al., 1970). mp 112–114°C. $[\alpha]_D^{24} -138.5°$ (c = 0.2, $CHCl_3$). IR ($CHCl_3$) no OH, 1760, 1710 (COO), 1635 (C = C-H) $cm^{-1}$. UV (EtOH) 222 nm ($\epsilon$ = 15300). EI-Ms *m/z*: 587 (M + 1), 513, 332.

*RLCC Separation of Fresh Methanol Extract.* The RLCC separation was performed on a model RLCC-A (Tokyo Rikakikai Co., Tokyo, Japan). The solvent system of $CHCl_3$–$CH_3OH$–$H_2O$ (13 : 7 : 4, v/v) in the ascending method was chosen. The methanol extract (1.0 g) was dissolved in the mobile phase and injected into the RLCC apparatus using a 3-ml sample chamber. The eluent was collected in 1.4-ml fractions which were monitored by TLC. The RLCC chromatogram is shown in Figure 4.

*HPLC Analyses.* Fresh leaves, fruits, and seeds (each amount weighed about 30 mg) were extracted with methanol for one week. After filtration and solvent removal, the methanol extract of each was passed through a SEP-PAC $C_{18}$ cartridge (for rapid sample preparation, Water Associates, Inc.) with $CH_3OH$–$H_2O$ (6 : 4, v/v) in order to remove some of the lipophilic portion of the extract. After evaporation of the solvent in vacuo, 10 $\mu$g of each residue was dissolved in methanol for injection into a HPLC apparatus (DuPont model 850 liquid chromatograph; DuPont Zorbax ODS, particle size 5–6 $\mu$m, 25 cm × 4.6 mm I.D). Compounds were detected by a DuPont variable-wavelength UV spectrophotometer and microflow cell at 254 nm using $CH_3OH$–$H_2O$ (6 : 4, v/v) as a solvent at a flow rate of 1.5 ml/min. The results are listed in Table 1.

*Antimicrobial Activity.* Compounds I, II, and III were screened for antimicrobial activity by the paper disk bioassay.

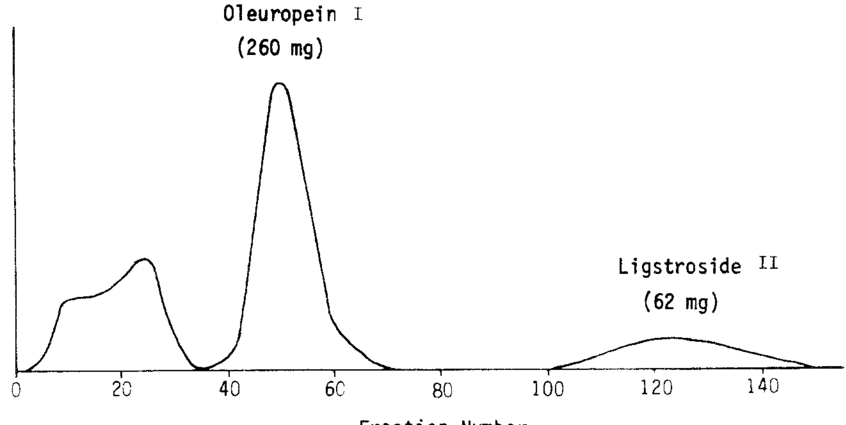


FIG. 4. RLCC of the methanol extract of *O. europaea* (1.0 g) with $CHCl_3$–$CH_3OH$–$H_2O$ (13:7:4, v/v) by the ascending method; 1.4 ml/fraction; 3 days.

Cultures of *S. cerevisiae* X2180–1B used in this study was obtained from Professor J. Thorner, and both *B. subtilis* and *E. coli* W3100 were obtained from Professor H. Nikaido, U.C. Berkeley.

Medium for *S. cerevisiae* was a 0.67% aqueous solution (20 ml) of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Michigan) containing 1.0% agar (Difco Laboratories), pH controlled by 1 N NaOH and 1 N HCl, and was autoclaved at 120°C for 15 min. After the solution was cooled to 50°C, it was seeded with a cell suspension of *S. cerevisiae* (a 24-hr culture at 30°C at pH 7 on a 0.67% aqueous solution of yeast nitrogen base without amino acids at a concentration of $10^4$ cells/ml). An aqueous solution (10 unit/ml) of $\beta$-glucosidase (Sigma Chemical Company, St. Louis, Missouri) was added to give a 0.5 unit/ml concentration in the medium. This mixture was then poured into a 90-mm-diameter Petri dish. The plating medium without added $\beta$-glucosidase was used as a control.

In the media for *B. subtilis* and *E. coli*, a 1% Pepton solution (Difco Lab-

TABLE 1.

| | Oleuropein I | | Ligstroside II | |
|---|---|---|---|---|
| | In fresh olive (%) | In MeOH ext (%) | In fresh olive (%) | In MeOH ext (%) |
| Fruit | 0.39 | 5.0 | 0.052 | 0.66 |
| Seed | 0.55 | 3.5 | 0.69 | 4.4 |
| Leaf | 0.42 | 3.5 | not found | not found |

oratories) was used instead of the yeast nitrogen base used for the medium of *S. cerevisiae*, and the cell suspensions of *B. subtilis* and *E. coli* were seeded at a concentration of $10^5$ cells/ml for each medium. Otherwise the medium was the same as for *S. cerevisiae.*

To test for antimicrobial activity by paper disk bioassay, appropriate volumes of the solutions were pipetted onto 6.5-mm-diameter paper disks to give desired dry weight quantities. Solutions were allowed to evaporate before the disks were placed on the seeded agar surface. Zones of inhibition were observed after incubation at 30°C for 16 hr. No zone of inhibition was observed in any case without added $\beta$-glucosidase. The results under the influence of $\beta$-glucodase are listed in Table 2.

*Oleanolic Acid (IV).* Colorless needles (Scheme 2). mp 300–302°C. $[\alpha]_D^{20}$ + 86.3° (c = 0.13, $CHCl_3$). IR (Nujol) 3400 (OH), 1700 (COOH) $cm^{-1}$. [$^1$H]NMR ($C_5D_5N$ + $CDCl_3$) $\delta$ 0.78, 0.87 (6H each, both S, OMe × 4), 0.94, 1.00, 1.16 (3H each, each s, OMe × 3), 2.79 (1H, dd, $J$ = 14 Hz, 3 Hz, 18-H), 3.20 (1H, d, $J$ = 8 Hz, 3-H), 5.28 (1H, t, $J$ = 3 Hz, 12-H). [$^{13}$C]NMR ($C_5D_5N$ + $CDCl_3$) $\delta$ 15.1 (q), 15.6 (q), 16.8 (q), 18.2 (t), 23.1 (t), 23.2 (t), 23.5 (q), 25.8 (q), 27.1 (t), 27.7 (t), 28.0 (q), 30.6 (s), 32.5 (t), 32.6 (t), 33.0 (q), 33.9 (t), 36.9 (s), 38.4 (t), 38.7 (s), 39.1 (s), 41.2 (d), 41.6 (s), 46.0 (t), 46.2 (s), 47.6 (d), 55.2 (d), 78.4 (d), 121.9 (d), 144.1 (s), 180.5 (s). EI-MS *m/z*: 456 ($M^+$), 423, 300, 203, 133, 119, 69.

*Maslinic Acid (V).* White powder (Scheme 2). mp 264–266°C. $[\alpha]_D^{20}$ + 60.0° (c = 0.01, $CHCl_3$). IR (Nujol) 3380 (OH), 1690 (COOH) $cm^{-1}$. [$^1$H]NMR ($C_5D_5N$ + $CDCl_3$) $\delta$ 0.80, 0.84, 0.92 (3H each, each s, OMe × 3), 0.92 (6H, s, OMe × 2), 1.05, 1.15 (3H each, both s, OMe × 2), 2.95 (1H, dd, $J$ = 14 Hz, 3 Hz, 18-H), 3.07 (1H, d, $J$ = 10 Hz, 3-H), 3.73 (1H, dt, $J$ = 10 Hz, 4 Hz, 2-H), 5.30 (1H, t, $J$ = 3 Hz, 12-H). [$^{13}$C]NMR ($C_5D_5N$ + $CDCl_3$) $\delta$ 16.4

IV : R=H, Oleanolic acid

V : R=OH, Maslinic acid

SCHEME 2.

TABLE 2. ANTIMICROBIAL SPECTRUM IN PRESENCE OF $\beta$-GLUCOSIDASE BY PAPER DISK BIOASSAY[a]

| | | Zone of inhibition (mm diameter) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Oleuropein I | | | Ligstroside II | | Dimethyl ester III | |
| Microorganism | (mg/disk) | 2.0 (mg/disk) | 1.0 (mg/disk) | 0.5 (mg/disk) | 1.0 (mg/disk) | 0.5 (mg/disk) | 1.0 (mg/disk) | 0.5 (mg/disk) |
| *Bacillus subtilis* | pH 8 | trace | —[b] | — | 8 | 7 | — | — |
| | pH 7 | 14 | 11 | 8 | 16 | 12 | 10 | trace |
| | pH 5 | NG[c] | NG | NG | NG | NG | NG | NG |
| *Saccharomyces cerevisiae* | pH 8 | 25 | 20 | 14 | — | — | — | — |
| | pH 7 | 16 | 13 | — | — | — | — | — |
| | pH 5 | — | — | — | — | — | — | — |
| *Escherichia coli* | pH 8 | — | — | — | — | — | — | — |
| | pH 7 | — | — | — | — | — | — | — |
| | pH 5 | — | — | — | — | — | — | — |

[a]No zone of inhibition was observed in any case without added $\beta$-glucosidase.
[b]— indicates no zone of inhibition.
[c]NG indicates that the bacteria did not grow in the medium.

(q), 16.7 (q, C × 2), 18.2 (t), 23.0 (t), 23.3 (t), 23.5 (q), 25.8 (q), 27.6 (t), 28.5 (q), 30.6 (s), 32.5 (t, C × 2), 33.0 (q), 33.9 (t), 38.1 (s), 39.0 (t), 39.2 (s, C × 2), 41.1 (d), 41.6 (s), 46.2 (t), 46.3 (s), 47.6 (d), 55.2 (d), 77.3 (d), 83.4 (d), 121.7 (d), 144.1 (s), 180.5 (s). EI-MS *m/z*: 472 ($M^+$), 426, 408, 300, 248, 203, 133, 119, 69.

## RESULTS AND DISCUSSION

The preliminary antimicrobial screening of the methanol extract of the fruits of *O. europaea* showed activity against *B. subtilis* (the fruits had been kept in methanol more than six months). Testing after separating the crude extract into ether-, ethyl acetate-, and water-soluble fractions indicated the active components were in the ether fraction. Attempts to isolate the active components in the ether fraction failed. We then examined the biological activity of the main compounds found in the methanol extract.

The $R_f$ values of the main compounds were determined to be 0.3–0.5 on a silica gel TLC plate using an organic layer of $CHCl_3$–$CH_3OH$–$H_2O$ (13:7:4, v/v) mixture. Due to its polar nature, the methanol extract seemed ideally suited for further separation by countercurrent chromatography which has been previously applied to the resolution of many polar mixtures (Hostettmann, 1980; Kubo et al., 1983). The initial DCCC attempt using $CHCl_3$–$CH_3OH$–$H_2O$ (13:7:4, v/v) by the ascending method was able to isolate compound I, but left II and III as a mixture (Figure 1). Reinjecting the mixture of II and III, and changing the solvent system to $C_6H_6$–$CHCl_3$–$CH_3OH$–$H_2O$ (5:5:7:2, v/v) in the ascending method resulted in baseline separation of II and III (Figure 2).

Structural determination by $[\alpha]_D$, UV, IR, MS, $[^1H]$- and $[^{13}C]$NMR showed I and II to be the known bitter *seco*-iridoid glycosides oleuropein (Panizzi et al., 1960) and ligstroside (Asaka et al., 1972). This was confirmed by spectral studies ($[\alpha]_D$, UV, IR, MS, NMR) of their acetylated derivatives Ia and IIa. This is the first isolation of ligstroside from olive.

The structure of III was shown by $[^1H]$- and $[^{13}C]$NMR to have the same *seco*-iridoid skeleton found in I and II, but the 3,4-dihydroxyphenylethyl of I and the 4-hydroxyphenylethyl group of II were replaced by a methyl group (Figure 3). The acetylated compound of III (IIIa) was identified by a direct comparison with an authentic sample (Inoue et al., 1970) based on physical and spectral data (mp, $[\alpha]_D$, IR, UV, EI-MS).

To see if compound III was an artifact arising from prolonged storage in methanol, fresh olive fruits were extracted, and the components were separated by rotation locular countercurrent chromatography (RLCC) (Kubo and Matsumoto, 1984. The RLCC separation took two days less than the DCCC separation and showed that compound III was an artifact that did not exist in the fresh fruit (Figure 4). HPLC was used to quantify the amounts of oleuropein (I) and ligs-

troside (II) in the fresh fruit body, seed, and leaf. The amounts of I and II from fresh and stored materials are listed in Table 1. No seasonal variation of these components was observed on a monthly basis over the period of a year. Oleuropein and ligstroside were found in the seed; I was the major compound in fruit and leaf (II was not detected in the leaf).

The presence of antimicrobial compounds in olive has been suspected for some time. Walter et al. (1973) and Fleming et al. (1973) reported the antimicrobial activity of oleuropein and its enzymatic hydrolysis poduct against bacteria and yeast. However, not enough data were available to identify the chemical structure of the enzymatic degradation product, nor could they examine the antimicrobial activity of the pure compound.

Cruess and Alsberg (1934) suggested that a much more active hydrolytic enzyme than emulsin was found in olive, and we examined the antimicrobial activity of I, II, and III with and without $\beta$-glucosidase (Table 2). Compounds I, II, and III showed no activity without $\beta$-glucosidase against *S. cerevisiae, B. subtilis,* and *E. coli* by the paper disk method. However, they inhibited the growth of *B. subtilis* at pH 7 in the presence of $\beta$-glucosidase. Oleuropein also inhibited the growth of *S. cerevisiae* at pH 7 and 8 with the enzyme (this conflicts with the results by Fleming et al., 1973). The data in Table 2 suggest, in the case of oleuropein, the active form is the aglycone hemiacetal (i) or the corresponding aglycone cleaved enal-aldehyde structure (ii) (Taniguchi et al., 1983), which is theoretically possible (Sticher, 1969) (Figure 5). Possibly the crude ether extract showed the antimicrobial activity previously mentioned because of the active aglycone, which would be easy to extract with ether but difficult to isolate because of its reactivity and instability.

Another defense mechanism against microbe attack of the olive leaf is a physical barrier on the leaf surface. The scanning electron micrographs show the leaf surface of *O. europaea* to be covered with crystals (Figure 6) (Kubo and Matsumoto, 1984). These crystals were carefully collected using a microscope and spatula without damaging the leaf's cuticle. TLC analysis ($CHCl_3$–$CH_3OH$, 20:1, v/v, silica gel) showed these contained a major and a minor component.

i ii

FIG. 5.

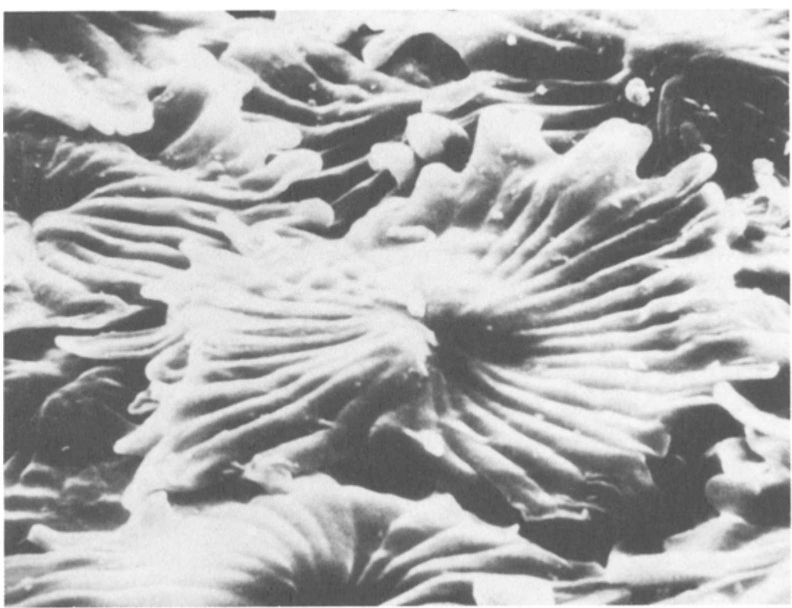

FIG. 6. The electron micrographs of the abaxial surface of the leaf of *O. europaea* (×550).

Purification by silica gel column chromatography gave two triterpenes. The major component was identified as oleanolic acid (IV, $C_{30}H_{48}O_3$, mp 300–302°C, $[\alpha]_D$ + 86.3°) from spectroscopic data (Roncero and Janer, 1969; Romualda et al., 1974). The very minor compound (V, $C_{30}H_{48}O_4$, mp 264–266°C, $[\alpha]_D$ + 60.0°) was identified by spectroscopic data as maslinic acid (Roncero and Janer, 1969; Romualda et al., 1974). The total yield of these triterpenes from the leaf surface (3.2% by weight) is comparable to the amount found for the whole leaf reported by Roncero and Janer (1969). Thus almost all oleanolic acid present is on the surface of the leaf. Oleanolic acid does not exhibit antimicrobial activity. However, this hydrophobic barrier on the leaf surface keeps water from collecting on the leaves and in this way inhibits the germination of microbes. This material also forms a physical barrier that might keep microbes from penetrating the leaf. This simple mechanism of secreting triterpenes onto the leaf surface must be considered as a part of a multichemical defense.

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# DOUBLE-BOND LOCATION IN MONOUNSATURATED FATTY ACIDS BY DIMETHYL DISULFIDE DERIVATIZATION AND MASS SPECTROMETRY: Application to Analysis of Fatty Acids in Pheromone Glands of Four Lepidoptera

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**Abstract**—A rapid analytical procedure for the determination of the position of double bonds in mixtures of monounsaturated fatty acid methyl esters has been developed. The method is based on direct capillary GC-MS-EI analysis of dimethyl disulfide adducts. The procedure was applied to mixtures of monounsaturated fatty esters from pheromone gland extracts of three tortricids from the *Choristoneura* genus, *C. fumiferana, C. occidentalis,* and *C. pinus pinus*, and one noctuid from the Plusiinae subfamily, *Plusia chalcites*. A correlation was found between the known major pheromone components in the four species with the corresponding fatty acids. Some of the additional fatty acids may be precursors to as yet unidentified minor pheromone components, present in extremely small quantities, in these species.

**Key Words**—Fatty acid analysis, double-bond location, dimethyl disulfide adducts, mass spectrometry, pheromone, *Choristoneura fumiferana*, *C. occidentalis*, *C. pinus pinus*, *Plusia chalcites*, Lepidoptera, Tortricidae, Noctuidae.

## INTRODUCTION

Numerous female-produced sex pheromone components have been identified in many families of Lepidoptera over the last 25 years (Inscoe 1982). A very large proportion of these compounds are $C_{10}$–$C_{18}$ monounsaturated acetates, alcohols,

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and aldehydes. In many instances, only the major pheromone components have been identified, often because very small quantities of pheromone are emitted and/or stored in the pheromone gland. Synthetic pheromone blends are rarely equivalent to the female-emitted pheromone in eliciting normal male precopulatory behaviors; often this is because additional (often minor in quantity) pheromone components have been overlooked primarily because of the type of bioassay used.

Recently, Bjostad and Roelofs (1981, 1983) demonstrated that fatty acid precursors of the female sex pheromone can be identified and used to predict the presence of minor pheromone components (Bjostad and Roelofs, 1983; Bjostad et al., 1984). Standard methods for double-bond location in lepidopteran sex pheromones involve isolation followed by ozonolysis, epoxidation, etc. (Beroza and Bierl, 1967; Bierl-Leonhardt et al., 1980). The present method (Bjostad et al., 1981; Bjostad and Roelofs, 1983) for the identification of monounsaturated pheromone gland fatty acid precursors involves the isolation of methyl esters by preparative gas chromatography, followed by ozonolysis and GC-MS of the products for location of the double bonds. This procedure is tedious, requires relatively large amounts of material, and would be complicated when a large number of positional isomeric fatty acids are present.

This paper reports a rapid analytical procedure for the location of double bonds in monounsaturated fatty acids obtained from extracts of the pheromone glands of four moth species. The method is based on the direct capillary GC-MS-EI analysis of the dimethyl disulfide (DMDS) adducts of fatty acid methyl esters similar to that applied to monounsaturated acetates (Buser et al., 1983). The moth species investigated, for which published information is available on their respective sex pheromone components, were three tortricids from the *Choristoneura* genus: *C. fumiferana* (Sanders and Weatherston, 1976; Silk et al., 1980), *C. occidentalis* (Cory et al., 1982; Silk et al., 1982), and *C. pinus pinus* (Silk et al., 1985), and one noctuid from the Plusiinae subfamily, *Plusia chalcites* (Dunkelblum et al., 1981).

## METHODS AND MATERIALS

*Insect and Gland Extracts.* The budworms *C. fumiferana, C. occidentalis,* and *C. pinus pinus* were reared on a synthetic diet (McMorran, 1965; Grisdale, 1970). Their ovipositor tips were excised by severing the abdomen just anterior to the pheromone gland during their "calling" period: for *C. fumiferana,* 2–4 days old, at the onset of scotophase (±1 hr) (Sanders, 1971); for *C. pinus pinus,* 1–4 days old, 2–4 hr into scotophase (Sanders, 1971); and for *C. occidentalis* (Silk et al., 1982), 2–4 days old, 1–2 hr prior to scotophase. *P. chalcites* were reared on artificial diet (Shorey and Hale, 1965), and tips of 4- to 6-day-old females were cut 1–3 hr into scotophase (Dunkelblum et al., 1981). Tips (10–

50) were soaked either in hexane or chloroform–methanol (2:1) for 24 hr and these extracts were used without further purification.

*Derivatization.* All derivatization reactions were performed in 1.5-ml conical vials with Teflon-lined screw caps. Extracts in hexane, which contained free fatty acids, were treated at room temperature with excess diazomethane in ether to convert the acids into methyl esters. Extracts in chloroform–methanol, which contained free and bound fatty acids, were submitted to acid methanolysis. Samples were concentrated under a stream of dry nitrogen to a volume of ca. 10 μl; 5% HCl (gas) in methanol (150 μl) was then added, and the reaction mixture was kept at 65–70°C (water bath) for 4 hr. After cooling, most of the methanol was evaporated with a stream of nitrogen, 250 μl hexane was added, and the mixture was washed with distilled water. The organic phase was removed, dried over $Na_2SO_4$, and concentrated to ca. 10 μl. One drop of acetyl chloride was added and the reaction mixture kept at room temperature for 15 min. Solvent and excess reagent were evaporated, and the products were dissolved in 10–20 μl hexane. Some samples were analyzed without acetylation.

Dimethyl disulfide (DMDS) adducts were prepared from the following standards (100–1000 ng); (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac), 11-dodecenyl acetate (11–12:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac), (*E*)- and (*Z*)-11-tetradecenyl acetate (*E*11–14:Ac, *Z*11–14:Ac); (*E*)- and (*Z*)-11-tetradecenol (*E*11–14:OH, *Z*11-14:OH), (*E*)- and (*Z*)-11-tetradecenal (*E*11–14:Ald, *Z*11–14:Ald), methyl-11-dodecenoate (11–12:Me), (*Z*)-9-tetradecenoate (*Z*9–14:Me), (*E*)- and (*Z*)-11-tetradecenoate (*E*11–14:Me, *Z*11–14:Me), (*Z*)-7-hexadecenoate (*Z*7–16:Me), (*E*)- and (*Z*)-9-hexadecenoate (*E*9–16:Me, *Z*9–16:Me), and (*Z*)-9-octadecenoate (*Z*9–18:Me).

Biological samples, methylated hexane extracts, or methanolysis products, were submitted to the same procedure. Samples in hexane (20–50 μl) were treated with 70–100 μl DMDS (Aldrich, gold label) and one drop of iodine solution (60 mg iodine in 1 ml diethyl ether). Reaction mixtures were kept at 40°C in a GC oven for 24 hr, cooled, and diluted with hexane (ca. 200 μl). Iodine was removed by shaking with 5% aqueous $Na_2S_2O_3$ (ca. 100 μl). The organic phase was removed and the aqueous phase extracted with 100 μl hexane. The combined hexane solution was dried over $Na_2SO_4$ and subsequently concentrated to a small volume (10–20 μl) and kept at 4°C.

*Gas Chromatography-Mass Spectrometry (GC-MS).* A Finnigan 4021 EI-CI system, at 70 eV, coupled with an INCOS data system was used for all GC-MS-EI analyses. A Grob injector was used for the capillary WCOT columns and was operated in the splitless mode with helium as carrier gas. For the analysis of fatty acid methyl esters and pheromonal compounds, columns A and B were used: A, FFAP, 50 m × 0.2 mm, at 50 °C for 4 min and then heated at 25°C/min to 200°C; B, DX4 (bonded Carbowax 20 M) 30 m × 0.32 mm, at 50°C for 2 min and then heated at 20°C/min to 180°C. For DMDS adducts, column C was used: C, DB5 (bonded SE-54) 30 m × 0.32 mm, at 50°C for 2

min and then heated at 30°C/min to 250°C. MS acquisition was started after 5 min for fatty esters and 10 min for DMDS adducts. With standards, the detection limit was in the range of 20–40 ng.

## RESULTS

The DMDS derivatives of monounsaturated fatty acid methyl esters were formed by the addition of DMDS to the double bond as described for alkenes (Francis and Veland, 1981) and monounsaturated acetates (Buser et al., 1983). The mass spectra of the derivatives exhibit four diagnostic mass peaks according to Scheme 1. The relative abundance of $M^+$ was in the range of 10–30% relative to the highest peak in the spectrum, whereas fragments $A^+$, $B^+$, and sometimes $C^+$ were above 50%. The DMDS addition to monounsaturated alcohols and aldehydes proceeded in the same manner; the mass spectra of adducts exhibit only three diagnostic mass peaks: $M^+$, $A^+$, and $B^+$. For illustration, the mass spectra of DMDS adducts of two isomeric methyl esters (Figure 1), and alcohol and aldehyde (Figure 2) are presented.

The analysis of fatty acid methyl esters from the three tortricids and the noctuid revealed an abundance of saturated and unsaturated esters. Extracts from all four species contained methyl dodecanoate (12:Me), tetradecanoate (14:Me), hexadecanoate (16:Me) and octadecanoate (18:Me) in varying amounts; in some samples methyl eicosanoate (20:Me) and docosanoate (22:Me) were detected. Also common to all four species were large quantities of $\Delta 18$:Me, $\Delta^2 18$:Me, and $\Delta^3 18$:Me, probably methyl oleate, linoleate, and linolenate, respectively. The chromatographic traces of the methylated extracts

$$CH_3(CH_2)_mCH{=}CH(CH_2)_nCH_2\overset{O}{\overset{\|}{C}}OCH_3 + CH_3SSCH_3 \xrightarrow{I_2} CH_3(CH_2)_m\overset{SCH_3}{\overset{|}{C}}H{-}\underset{\underset{SCH_3}{|}}{C}H(CH_2)_nCH_2\overset{O}{\overset{\|}{C}}OCH_3$$

$$\xrightarrow{MS\text{-}EI} \left[CH_3(CH_2)_m\overset{SCH_3}{\overset{|}{C}}H{-}\underset{\underset{SCH_3}{|}}{C}H(CH_2)_nCH_2\overset{O}{\overset{\|}{C}}OCH_3\right]^{+} \cdot M^+$$

$$\underset{A^+}{CH_3(CH_2)_m\overset{^+SCH_3}{\overset{\|}{C}}H} \qquad \underset{B^+}{\underset{\underset{^+SCH_3}{\|}}{C}H(CH_2)_nCH_2\overset{O}{\overset{\|}{C}}OCH_3} \xrightarrow{-CH_3OH} \underset{C^+}{\underset{\underset{^+SCH_3}{\|}}{C}H(CH_2)_nCH{=}C{=}O}$$

SCHEME 1.

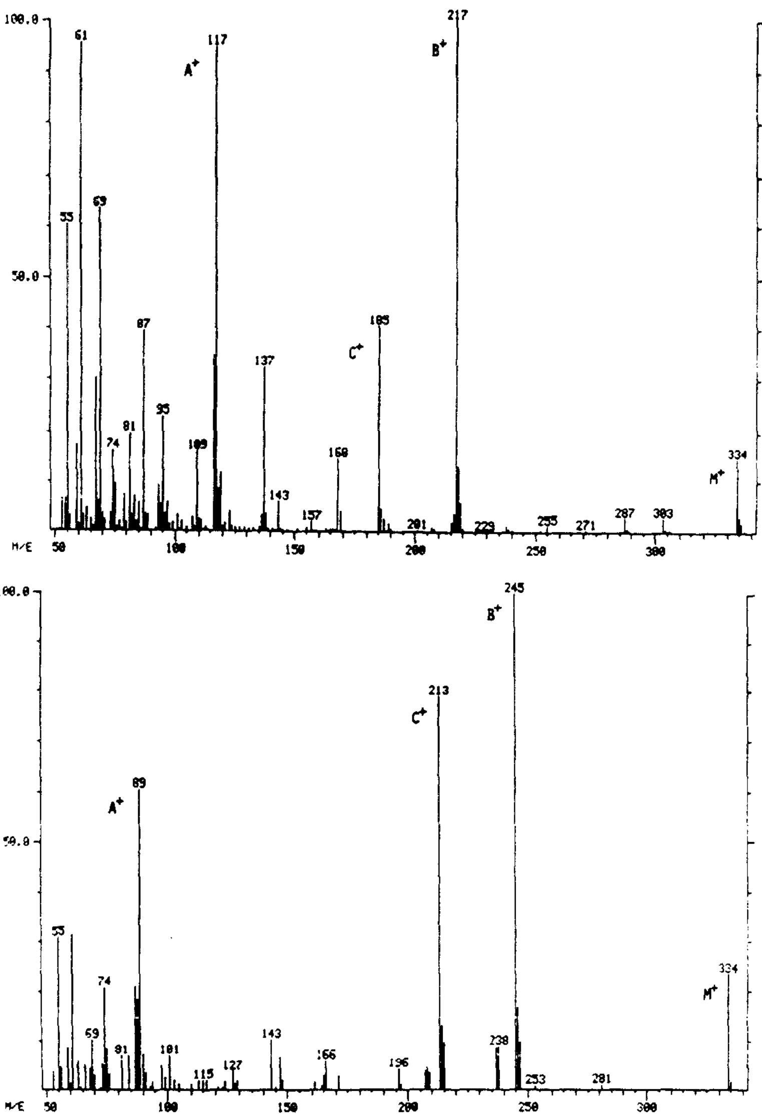


FIG. 1. Mass spectra of DMDS adducts of methyl (*Z*)-9-tetradecenoate (top) and methyl (*E*)-11-tetradecenoate (bottom).

from the three budworms were very similar, showing clearly and specifically at least one methyl Δ-tetradecenoate (Δ14 : Me) and three isomeric methyl Δ-hexadecenoates (Δ16 : Me); no Δ-dodecenoates (Δ12 : Me) were detected. On the other hand, the extract from *P. chalcites* contained large amounts of methyl Δ-dodecenoates (Δ12 : Me), Δ14 : Me, two Δ16 : Me esters, and a small amount of

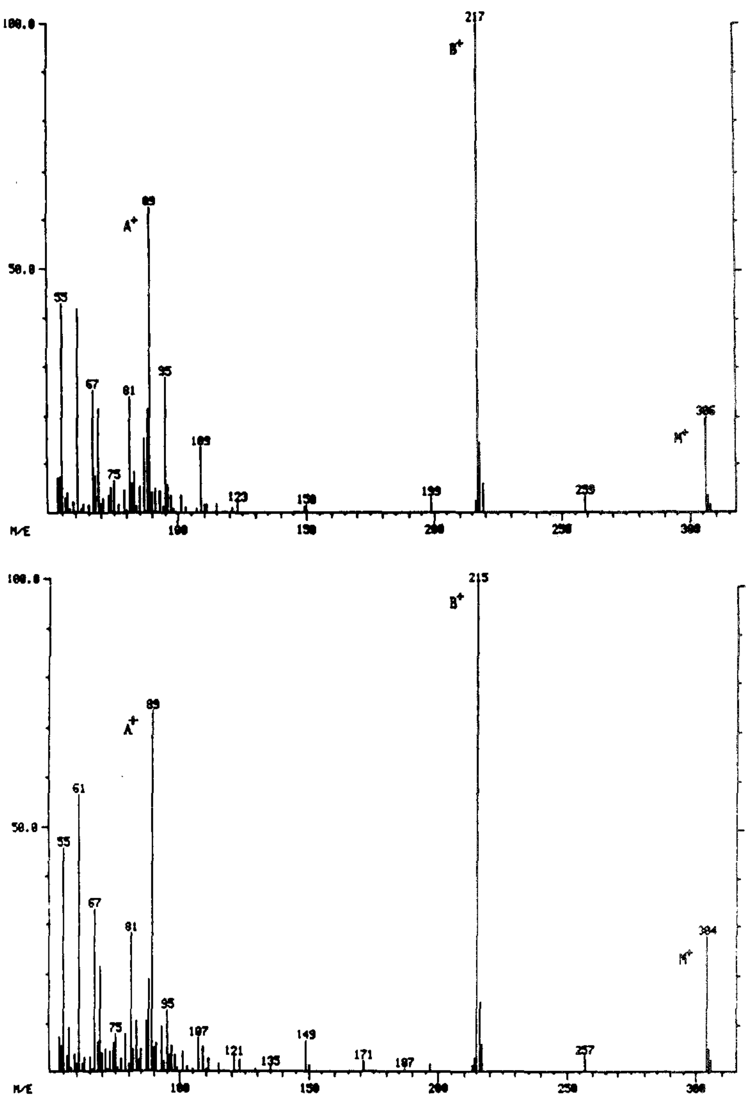


FIG. 2. Mass spectra of DMDS adducts of ($E$)-11-tetradecenol (top) and ($E$)-11-tetradecenal (bottom).

an additional Δ18 : Me ester. All extracts contained some pheromone components and hydrocarbons. The total ion chromatograms from *C. fumiferana* and *P. chalcites* are presented in Figure 3.

For the location of the double bonds in the monounsaturated fatty esters in the extracts, DMDS derivatization was used, and the crude reaction mixtures were analyzed by GC-MS-EI on column C. Monitoring of appropriate ions $A^+$

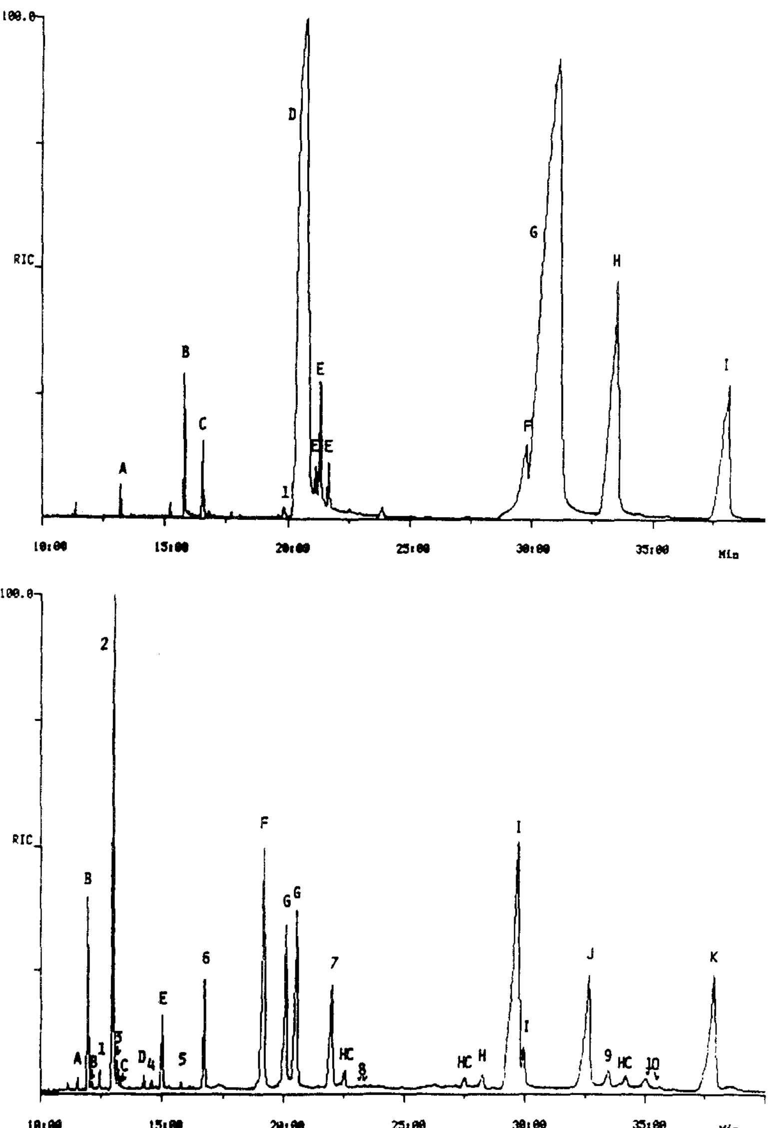


FIG. 3. Total ion chromatograms of methanolyzed *Choristoneura fumiferana* ovipositor extract (top) and methylated hexane *Plusia chalcites* ovipositor extract (bottom) analyzed on column A. The components for *C. fumiferana* were: (A) 12:Me, (B) 14:Me, (C) Δ14:Me, (1) *E*11-14:OH, (D) 16:Me, (E) Δ16:Me, (F) 18:Me, (G) Δ18:Me, (H) $\Delta^2$18:Me, (I) $\Delta^3$18:Me; and for *P. chalcites:* (A) 12:Me, (B) Δ12:Me, (1) 12:Ac, (2) *Z*7-12:Ac, (3) *Z*9-12:Ac + 11-12:Ac, (C) Δ13:Me, (D) 14:Me, (4) Δ13:Ac, (E) Δ14:Me, (5) 14:Ac, (6) *Z*9-14:Ac, (F) 16:Me, (G) Δ16:Me, (7) 16:Ac, (8) Δ16:Ac, (H) 18:Me, (I) Δ18:Me, (J) $\Delta^2$18:Me, (9) 18:Ac, (10) Δ18:Ac, (K) $\Delta^3$18:Me, (HC) hydrocarbon.

and $B^+$ was applied for detection of minor components. Two typical total ion chromatograms of DMDS reaction mixtures of *C. fumiferana* and *P. chalcites* samples are presented in Figure 4, showing peaks in the $\Delta$12 : Me · DMDS–$\Delta$16 : Me · DMDS region. In each of the four insects studied, $\Delta$9–18 : Me · DMDS was the most abundant fatty ester adduct present. However, in order not to distort the chromatograms, this region is not shown in Figure 4. The additional $\Delta$18 : Me present only in *P. chalcites* was identified as $\Delta$11–18 : Me.

The mass spectrometric data of the DMDS derivatized monounsaturated fatty esters from the four species is summarized in Table 1. The $\Delta^2$18 : Me and $\Delta^3$18 : Me esters could not be detected after derivatization. These polyunsaturated esters were either polymerized by DMDS or the di and tri adducts were not sufficiently volatile to elute from the GC column. The monounsaturated fatty methyl esters ($C_{12}$–$C_{16}$) detected by the DMDS method for the four species analyzed are summarized in Table 2. The relative amounts could only be roughly estimated because some of the DMDS esters were found in extremely small quantities while others appeared as clusters of isomers only partially separated.

The GC-MS chromatograms of the derivatized samples also revealed the presence of pheromone components. In the case of the three budworms, $\Delta$11–14 : Ac (*E*/*Z* pair) was always present in the glands (Silk and Kuenen, 1984, and references therein) or the corresponding alcohol if the methanolysis was not followed by reacetylation. In addition, trace amounts of hexadecenyl acetates ($\Delta$16 : Ac) were detected. The total amount of pheromonal components in the budworms is very low, therefore, on an average injection only the major compound *E*11–14 : Ac could be found. In order to detect the extremely small amounts of the corresponding *Z* isomer and the $\Delta$16 : Ac, large amounts of material were introduced into the GC-MS. In the case of *P. chalcites,* a series of $\Delta C_{12}$–$\Delta C_{18}$ acetates was found. The derivatized monounsaturated acetates in *P. chalcites* are listed in Table 3.

## DISCUSSION

It has recently been demonstrated that specific monounsaturated fatty acid moieties are precursors to monounsaturated pheromone components in moths (Bjostad and Roelofs, 1981, 1983; Bjostad et al., 1981). Being more abundant than the pheromone components, these possible precursors could be helpful in the search for minor pheromone components which often are extremely difficult to detect. Despite the fact that the fatty acids can be easily converted into methyl esters and analyzed by GC, the location of the double bond is still a difficult problem. DMDS derivatization of the monounsaturated fatty acid esters and direct analysis by GC-MS-EI overcomes these difficulties and provides an excellent complementary method to study complex mixtures of positional isomers.

The compounds investigated gave very distinct EI mass spectra which display diagnostic fragments allowing the location of double bonds. The reaction

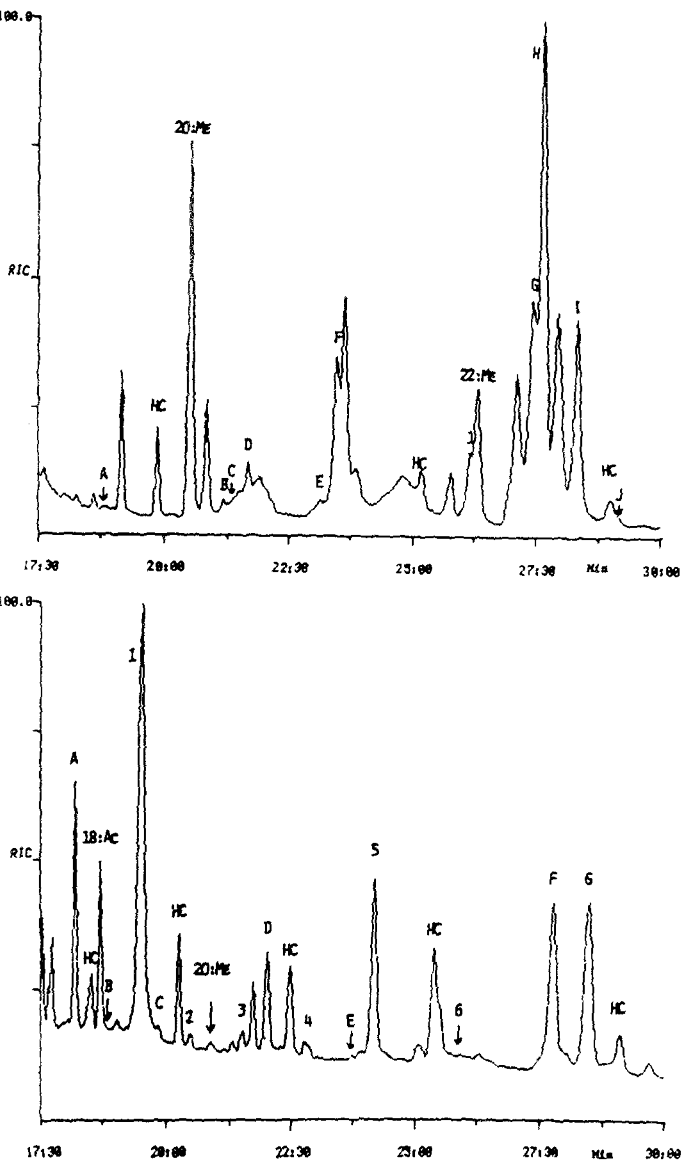


FIG. 4. Total ion chromatograms of DMDS adducts of methanolyzed and acetylated *Choristoneura fumiferana* ovipositor extract (top) and methylated hexane *Plusia chalcites* ovipositor extract (bottom) analyzed on column C. The components for *C. fumiferana* were: (A) Δ9–12 : Me, (B) Δ5–14 : Me, (C) Δ7–14 : Me, (D) Δ9–14 : Me, (E) *Z*11–14 : Me, (F) *E*11–14 : Me, (1) *E*11–14 : Ac, (G) Δ7–16 : Me, (H) Δ9–16 : Me, (I) Δ11–16 : Me, (J) Δ12–16 : Me; and for *P. chalcites:* (A) Δ7–12 : Me, (B) Δ9–12 : Me, (1) *Z*7–12 : Ac, (C) Δ8–13 : Me, (2) Δ9–12 : Ac, (3) Δ8–13 : Ac, (D) Δ9–14 : Me, (4) 11–12 : Ac, (E) Δ11–14 : Me, (5) *Z*9–14 : Ac, (6) Δ11–14 : Ac, (F) Δ9–16 : Me, (G) Δ11–16 : Me.

TABLE 1. MASS SPECTROMETRIC DATA OF MONOUNSATURATED FATTY ESTER DMDS DERIVATIVES IN OVIPOSITOR EXTRACTS FROM THREE *Choristoneura* SPECIES AND *P. chalcites*

| DMDS of Δ-fatty esters | Diagnostic ions $M^{+a}$ | $A^+$ | $B^+$ | $C^+$ | Insect[b] | | | |
|---|---|---|---|---|---|---|---|---|
| 7-12:Me | 306 | 117 | 189 | 157 | | | | Pc |
| 9-12:Me | 306 | 89 | 217 | 185 | Cf | Co | Cpp | Pc |
| 8-13:Me | 320 | 117 | 203 | 171 | | | | Pc |
| 5-14:Me | 334 | 173 | 161 | 129 | Cf | Co | Cpp | |
| 7-14:Me | 334 | 145 | 189 | 157 | Cf | Co | Cpp | |
| 9-14:Me | 334 | 117 | 217 | 185 | Cf | Co | Cpp | Pc |
| 11-14:Me | 334 | 89 | 245 | 213 | Cf | Co | Cpp | Pc |
| 7-16:Me | 362 | 173 | 189 | 157 | Cf | Co | Cpp | |
| 9-16:Me | 362 | 145 | 217 | 185 | Cf | Co | Cpp | Pc |
| 11-16:Me | 362 | 117 | 245 | 213 | Cf | Co | Cpp | Pc |
| 12-16:Me | 362 | 103 | 259 | 227 | Cf | Co | | |
| 9-18:Me | 390 | 173 | 217 | 185 | Cf | Co | Cpp | Pc |
| 11-18:Me | 390 | 145 | 245 | 213 | | | | Pc |

[a] In some cases the molecular ion $M^+$ was absent in the minor components.
[b] Cf = *C. fumiferana;* Co = *C. occidentalis;* Cpp = *C. pinus pinus;* Pc = *P. chalcites.*

TABLE 2. MONOUNSATURATED FATTY ESTER DMDS DERIVATIVES IN OVIPOSITOR EXTRACTS FROM THREE *Choristoneura* SPECIES AND *P. chalcites*

| DMDS Δ-fatty esters[a] | *C. fumiferana*[b] | *C. occidentalis*[b] | *C. pinus pinus*[b] | *P. chalcites*[c] |
|---|---|---|---|---|
| 7-12:Me | | | | L |
| 9-12:Me | T | T | T | VS |
| 8-13:Me | | | | VS |
| 5-14:Me | VS | VS | T | |
| 7-14:Me | T | T | T | |
| 9-14:Me | VS | VS | VS | M |
| 11-14:Me | M | L | S | T |
| 7-16:Me | M | M | S | |
| 9-16:Me | L | L | L | L |
| 11-16:Me | M | M | S | L |
| 12-16:Me | T | VS | | |

[a] Quantitative estimate relative to the most abundant fatty ester DMDS adduct ($C_{12}$–$C_{16}$) L 100–50; M 50–30; S 30–10; VS 10–1; T <1 (L, M, S, VS, T: large, medium, small, very small, and trace, respectively; in %). All four insects contain very large amounts of Δ9-18:Me DMDS and only *P. chalcite* has some Δ11-18:Me DMDS.
[b] Methanolyzed samples of chloroform–methanol extracts.
[c] Methylated samples of hexane extract.

TABLE 3. RELATIVE PROPORTIONS OF MONOUNSATURATED ACETATE DMDS DERIVATIVES IN OVIPOSITOR TIP EXTRACTS OF *P. chalcites*

| Double bond | 12:Ac | 13:Ac | 14:Ac | 16:Ac | 18:Ac |
|---|---|---|---|---|---|
| Δ7 | 100[a] | | | | |
| Δ8 | | 2–3 | | | |
| Δ9 | 1–2[a] | | 18–20[a] | 1–3 | 2–4 |
| Δ11 | 1–2 | | <1 | 1–3 | 1–2 |

[a]Previously identified acetates (Dunkelblum et al., 1981).

is stereospecific for both *E* and *Z* isomers (checked with *E*- and *Z*11–14:Me. DMDS and *E*- and *Z*9–16:Me·DMDS), yielding one adduct in each case, with the *Z* adduct eluting first. The same elution characteristics were reported for the monounsaturated acetates adducts with the exception of Δ2 isomers (Buser et al., 1983). The mass spectra provide readily recognizable key fragments, including the molecular ion $M^+$ and fragments $A^+$ and $B^+$. One of these diagnostic fragments is often the base peak in the spectrum. In addition there is an important fragment $C^+$ formed from $B^+$ by loss of methanol ($C^+ = B^+ - 32$) (Figure 2). This fragment makes it possible to differentiate between a DMDS adduct derived from an acetate of a $C_n$ alcohol and a methyl ester of a $C_{n+1}$ acid with the double bond shifted by one position such as: Δ7–12:OAc·DMDS and Δ8–13:Me·DMDS. Both adducts have the same $M^+$, $A^+$, and $B^+$, but the $C^+$ fragments are different; for the acetate, $C^+ = B^+ - 60$; for the methyl esters, $C^+ = B^+ - 32$.

The spectra of the DMDS adducts of all fatty esters, standards as well as those from the insects, fit into this scheme (Table 1). When both adducts from *E* and *Z* geometrical isomers were present, the configuration of the olefinic bond was inferred from the fact that the *Z* adduct was the first one eluting. Since the *E*/*Z* ratio of fatty acid isomers of pheromone precursors is not directly correlated with the isomer ratio found in pheromone components (Bjostad et al., 1981), the critical information that accrues from this method is the position of the double bond. Therefore, only this information is recorded in Table 2. Some of the less abundant fatty esters were detected or characterized only as their DMDS adducts; these were Δ9–12:Me; Δ5, Δ7, and Δ9–14:Me and Δ12–16:Me for the three budworms and Δ11–14:Me as well as Δ11–14:Ac for *P. chalcites*. The excellent separation of positional isomeric DMDS adducts made it possible to distinguish readily between *Z*9–12:Ac and 11–12:Ac (Figure 4) which, underivatized, eluted as one peak close to *Z*7–12:Ac (Figure 3).

It was found that hexane extracts contained large quantities of free fatty acids. These acids were converted with diazomethane to methyl esters and these mixtures were compared for the three budworms with methanolysis mixtures

which contained free and bound fatty acids. The GC profiles were similar in each case, although the relative amounts of some acids were different. In the case of *P. chalcites,* only a hexane extract was available. In some extracts, in particular from *C. fumiferana,* a number of fatty acid methyl esters were found in hexane extracts prior to methylation.

The blends of the fatty acids from the three budworms were similar, differing only in the relative abundance of components, but they were completely different from the blend of *P. chalcites* (Table 2). Not all of the monounsaturated fatty esters are precursors of pheromone components; some, in particular Δ9-16 : Me and Δ9-18 : Me, are common fatty acids in natural lipids (Bjostad et al., 1981). However, the different specific blends from the *Choristoneura* species and *P. chalcites* indicate a possible correlation between certain fatty acids and appropriate pheromone components. The major pheromone components, which have been previously identified, are readily correlated with the corresponding fatty acids. In the budworms, these are the *E*/*Z*11-14 : Me with the *E*/*Z*11-14 : OH, Ald, and Ac (Silk and Kuenen, 1984). In *P. chalcites* these are Δ7-12 : Me, Δ9-12 : Me and Δ9-14 : Me with *Z*7-12 : Ac, *Z*9-12 : Ac, and *Z*9-14 : Ac (Dunkelblum et al., 1981). The other unsaturated fatty acids are possible precursors to yet unidentified pheromone components in these species.

Attempts to correlate some of these fatty acids with additional minor components in the budworms were not successful, and only traces of Δ16 : Ac were detected. This is probably due to the extremely low quantities of potential minor components; therefore analysis of extracts from large numbers of glands may be necessary. In the case of *P. chalcites,* which contains large amounts of pheromone (Dunkelblum et al., 1981), a good correlation between fatty acids and acetates was established (Tables 2 and 3), with the exception of 11-12 : Me which was not detected. The presence of Δ8-13 : Me and the corresponding acetate Δ8-13 : Ac appears to be novel in the Lepidoptera.

## CONCLUSIONS

Rapid location of the double bonds of monounsaturated fatty acids can be performed by capillary GC-MS-EI analysis of the DMDS adducts of their methyl esters. Analysis of three *Choristoneura* species and of *P. chalcites* showed different fatty acid profiles for the two groups. These fatty acids may predict additional pheromone components not found directly in effluvia or pheromone gland extracts (Bjostad and Roelofs, 1983; Bjostad et al., 1984). Further work is in progress to establish which of the potential minor pheromone components is significant in eliciting behavioral responses from males and may be of importance in future field applications.

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# EFFECTS OF FERULIC ACID, AN ALLELOPATHIC COMPOUND, ON LEAF EXPANSION OF CUCUMBER SEEDLINGS GROWN IN NUTRIENT CULTURE.[1]

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**Abstract**—Cucumber seeds and seedlings at various ages (7–19 days old) were treated with a single treatment or multiple treatments (at 2-day intervals) of ferulic acid in nutrient culture. Ferulic acid treatments of cucumber seeds during stages of germination and radicle growth did not significantly reduce subsequent seedling growth. Ferulic acid treatments to seedlings reduced leaf area, leaf expansion, and dry weight of cucumber seedlings. Pretreatment of seeds and seedlings with 0.1 or 0.2 mM ferulic acid did not modify the effects of a single 1 mM ferulic acid treatment on leaf expansion when a single treatment was given at various times to seedlings ranging from 7 to 19 days of age. Treatments of 1mM or greater induced rapid wilting of leaves, but visible recovery occurred within 24–48 hr and subsequent treatments did not cause wilting. Once seedlings were removed from ferulic acid treatments, leaf expansion resumed. The magnitude of recovery depended on the concentration of ferulic acid, frequency of ferulic acid application and age of the seedling. Mean relative rates of leaf expansion recovered rapidly even in the presence of ferulic acid. Recovery of leaf expansion after ferulic acid treatments was faster for seedlings grown in an adequate nutrient environment than for seedlings grown in a limited nutrient environment. Ferulic acid disappeared from nutrient solutions with time, and two microbial metabolic products of ferulic acid (i.e., vanillic and protocatechuic acid) were identified in nutrient solutions.

**Key Words**—Allelopathy, ferulic acid, cucumber seedlings, *Cucumus sativus*, absolute leaf expansion, relative leaf expansion.

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## INTRODUCTION

Higher plants produce a wide array of organic compounds, some of which ultimately end up in the soil environment and affect the growth and development of neighboring plants. The compounds that affect plant growth and development have been designated as allelopathic compounds (Rice, 1974, 1979). The magnitude of the impact of allelopathic compounds on plants will depend on the type of compound, the concentration and stability of that compound in the soil, and a plant's resistance and/or sensitivity to that compound. Ferulic acid is one of the allelopathic compounds that has been identified (Wang et al., 1967; del Moral and Muller, 1970; McPherson et al., 1971; Rassmussen and Einhellig, 1977; Patterson, 1981). It is commonly found in plants (Bates-Smith, 1956), has been isolated from soil (Whitehead, 1964; Guenzi and McCalla, 1966; Wang et al., 1967; Lodhi, 1975; Whitehead et al., 1981, 1982), and is a product of lignin degradation (Flaig, 1964; Turner and Rice, 1975; Martin and Haider, 1976).

Previously, we reported that cucumber radicle growth (*Cucumis sativus* cv 'Early Green Cluster') was reduced by ferulic acid and several of its microbial metabolic products (Blum et al., 1984). The present report is an extension of that research, being an effort to define and refine methodology that will aid in an understanding of allelopathic interactions between plants. The primary objectives were: (1) to determine if the observed inhibition of cucumber radicle growth by ferulic acid was simply a short-term suppression of growth or a suppression that could ultimately affect cucumber seedling growth, (2) to determine how time and frequency of application of various ferulic acid concentrations might affect cucumber seedling growth, (3) to determine if leaf area expansion of cucumber seedlings could be used as a reliable and rapid means of assessing inhibitory effects of ferulic acid, and (4) to determine if cucumber seedlings can be acclimated to ferulic acid. Although all these objectives address knowledge required for the development of adequate bioassay procedures, objectives (2) and (3) are of particular interest because background levels and input doses of allelopathic compounds in the soil environment are constantly changing (Dalton et al., 1983; Lodhi, 1976 and 1978). Since allelopathic interactions are concentration-dependent, a rapid indicator of plant response to allelopathic compounds would aid in the determination of mechanisms of action and provide a more precise way of describing plant behavior in the presence of changing concentrations of allelopathic compounds in soil environments. In addition, if the impact of ferulic acid on growth is reversible, then a rapid evaluation technique is essential.

## METHODS AND MATERIALS

*General Aspects.* Cucumber seeds (*Cucumis sativus* cv 'Early Green Cluster'; Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated

in the dark at 28–30°C in bowls or trays containing sterile vermiculite and Hoagland's solution. After 48 hr, the seedlings were transferred to 120-ml glass snap-cap bottles containing nutrient solution at pH 5.8. Seedlings were suspended in nutrient solution by a foam collar through a hole in the cap of the bottles. Solutions and roots in bottles were protected from direct irradiance by inserting the bottles through holes in a plywood sheet so that the translucent plastic snap-caps were flush with the upper surface of the plywood sheet. This surface was covered with white paper (except where perforated for the insertion of bottles). Aluminum foil panels were suspended on all four sides of the light banks so that the base of the panels was 5 cm above the plywood sheet and the top of the panels was 25 cm below the light source, thus providing adequate ventilation. Three light banks, each 60 × 125 cm and composed of 12, 40-watt cool white fluorescent tubes and six, 25-watt incandescent bulbs provided 189 $\mu$Einsteins/$m^2$/sec for 12 hr per day at the plywood sheet surface. The temperature just above the plywood surface under the light banks ranged from 21° to 30°C for all experiments. Experimental treatments were randomly distributed under each of the three light banks.

*Background Studies.* The following experiment was carried out to determine how the strength of the nutrient solution might affect dry weight production and leaf expansion of cucumber. Forty-eight hours after germination was initiated, seedlings were transferred to bottles containing either full, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ or $\frac{1}{64}$ strength Hoagland's solution (Hoagland and Arnon, 1938). For one set of plants (total number of plants = 21), solutions were changed every other day, while for the other set (total number of plants = 21) nutrient solutions were supplied once at the beginning (day 2). In the latter case, deionized water was added to the bottles every other day to replenish the water lost by transpiration. Leaf length and width were recorded every other day. On day 17 leaf area of individual true leaves (i.e., cotyledonary areas were excluded) was taken with a Li-cor model Li 3000 portable leaf area meter, and shoot and root dry weights (50°C, 48 hr) were obtained.

Since earlier investigators frequently supplied nutrient solution and phenolic acids only at the beginning of their experiments, one set of plants was treated in this manner. Seedlings were treated with 0, 0.5, 1, or 2 mM ferulic acid (Sigma Chemical Company, St. Louis, Missouri) in full strength Hoagland's solution when 7 days old (i.e., all solutions were changed). Subsequently, to half of the plants (total number of plants = 12) only water was added to the bottles every other day to replenish water lost by transpiration. The remaining plants (total number of plants = 12) were transferred to full-strength Hoagland's solution free of ferulic acid after 48 hr (i.e., day 9). Thereafter, nutrient solutions for these plants were changed every other day. Length and width of leaves were measured every other day; leaf area and dry weights were determined when plants were 17 days old.

In a preliminary experiment (data not presented), we observed that the

ferulic acid content in nutrient solutions containing cucumber roots of 14- to 16-day-old seedlings disappeared after 24–48 hr. To determine how this pattern of disappearance might be influenced by additional applications of ferulic acid, a cucumber plant was supplied with 1 mM ferulic acid in full-strength Hoagland's solution every two days starting with day 5. A new supply of ferulic acid solution was given every other day for a total of six periods. Concentrations of ferulic acid and/or its microbial and physical breakdown products in nutrient solutions were determined with a high-performance liquid chromatograph (Blum et al., 1984).

The number of cells per unit leaf area was determined from electron micrographs (EM) of both mature and developing leaves from seedlings (total number of plants = 6) treated with 0, 0.5, or 1 mM ferulic acid. The first ferulic acid treatment for plants in the EM study was applied at day 8. Solutions were changed every other day. Leaves were harvested when seedlings were 16 days old.

*Inhibition of Radicle Growth and Subsequent Seedling Growth.* This experiment was designed to determine if the observed inhibition of radicle growth by ferulic acid at 48 hr (Blum et al., 1984) was simply a short-term suppression of radicle growth or a more permanent injury that could ultimately affect cucumber seedling growth and development. Cucumber seeds were germinated in glass bowls containing vermiculite, full- or $\frac{1}{16}$-strength Hoagland's solution, and 0, 0.5, or 1.0 mM ferulic acid. After 48 hr, seedlings from each germination treatment were transferred to bottles containing either full- or $\frac{1}{16}$-strength Hoagland's solution and 0, 0.5 or 1.0 mM ferulic acid. Seedlings (total number of plants = 54) were then placed under the light banks. Solutions were changed every other day. Length and width measurements for all leaves were taken whenever solutions were changed. Dry weights of seedlings were determined when plants were 21 days old.

*Recovery after Treatment and Time of Initial Treatments.* These experiments were designed to determine how time and frequency of application of various ferulic acid concentrations might affect cucumber seedling growth. Seeds were germinated in glass bowls containing vermiculite and full-strength Hoagland's solution and were transferred to bottles containing full-strength Hoagland's solution. Solutions were changed every other day. In one experiment (total number of plants = 48), ferulic acid was added initially at day 7 to all treatments but the control. Subsequently, a set ($N$ = 3) of plants was removed from ferulic acid treatment every other day. In a second experiment, all seedlings (total number of plants = 48) were started in full-strength Hoagland's solution. A new set of seedlings was treated every other day starting with day 7. Once treatment was initiated, ferulic acid solutions were changed every other day. Thus these two experiments had inverse treatment regimes. Ferulic acid concentrations used were 0, 0.5, 1.0, and 2 mM. Leaf length and width were measured every other day, and dry weights of seedlings were determined on day 17.

To determine if the decline in seedling leaf area was due to the water potential of the ferulic acid solutions, plants (total number of plants = 9) were treated with either full-strength Hoagland's solution, Hoagland's solution plus 2 mM ferulic acid, or Hoagland's solution plus polyethylene glycol (6000 mol wt) when plants were 14 and 16 days old. The initial values of the solutes were 22 milliosmoles (mOsm) for the Hoagland's solution, 33 mOsm for the 2 mM ferulic acid solution, and 27 mOsm for the polyethylene glycol solution. Length and width measurements of leaves were made when seedlings were 14 and 16 days old. The volume of the solutions remaining in the bottles was also determined at that time. The initial volume in each bottle was 118 ml. The milliosmoles of the solutes were determined with an Osmette precision osmometer at day 16.

*Potential Acclimation.* This experiment (total number of plants = 72) was designed to determine whether cucumber seedlings may become acclimated to ferulic acid. Seeds were germinated in glass bowls containing vermiculite and 0, 0.1, or 0.2 mM ferulic acid in full-strength Hoagland's solution. After 48 hr, seedlings were removed to bottles containing 0, 0.1, or 0.2 mM ferulic acid in full-strength Hoagland's solution. At days 7, 9, 11, 13, 15, 17, and 19, a set of plants from each background treatment was given, in addition to the background level (0, 0.1, or 0.2 mM), a 1 mM treatment of ferulic acid. Plants were allowed to grow in these solutions for 48 hr before they were again placed into their background solutions. Nutrient solutions with or without the background levels were changed every other day. Length and width of leaves were measured every other day and plant dry weights were determined at day 21.

*Data Analysis.* All treatments for each experiment were randomly distributed under each of the three light banks. Unless otherwise stated, all experimental treatments had an $N$ value of 3. Data were analyzed using the Statistical Analysis System (SAS) programs for analysis of variance and linear and multiple regressions (Helwig and Council, 1979). Least significant differences ($LSD_{0.05}$), where presented, are provided only as a measure of experimental precision, and inferences are based on the appropriate analysis of variance or regression analysis.

The mean absolute rate of leaf expansion was defined by the equation:

$$\text{Mean absolute rate} = \text{leaf area at time}_{x+1} - \text{leaf area at time}_x$$

Since all readings were taken at 2-day intervals, values given are based on these 2-day periods. Mean relative rate of leaf expansion was defined by the equation:

$$\text{Mean relative rate} = \ln(\text{leaf area at time}_{x+1} + 1) - \ln(\text{leaf area of time}_x + 1)$$

As with absolute rates, these values were based on 2-day intervals. One was added to each value since zero leaf area values were not uncommon for the early growth periods and the logarithm of zero is undefined. For additional details about growth analysis equations see Radford (1967) or Evans (1972).

## RESULTS AND DISCUSSION

*Background Studies.* Since nutrient levels in the growth medium can modify the effects of phenolic acids on plant growth (Hall et al., 1983; Stowe and Osborn, 1980), we grew cucumber seedlings in various concentrations of Hoagland's solution to determine the nutrient strength necessary for good growth under the growth environment of the light banks. We chose to supply various nutrient solutions once at the beginning (day 2) to some plants and to change the nutrient solutions every other day for others. The former approach was taken because this had been the procedure commonly used by other investigators (e.g., Olmsted and Rice, 1970; Einhellig and Rasmussen, 1979; Lodhi, 1979). The latter procedure (i.e., changing solutions every other day) was used because we noted in preliminary experiments that a large portion of the ferulic acid supplied to 10- to 16-day-old cucumber seedlings disappeared from nutrient solution within 24–48 hr. This was most likely due to a combination of microbial metabolism, physical breakdown, and root adsorption and/or absorption of ferulic acid. Since we did not wish to confound nutrient and phenolic acid effects, additional applications of ferulic acid to seedlings were applied in fresh nutrient solutions each time (i.e., a complete solution change).

A full strength Hoagland's solution given once at the beginning (day 2) appeared to be adequate for maximum growth of cucumber seedlings under our light banks over the time period of this bioassay; changing full-strength Hoagland's solutions every other day did not modify the growth of cucumber seedlings (Table 1). We next determined if the effects of ferulic acid might be different for single and multiple applications of nutrient solution. The effects of

TABLE 1. LEAF AREA AND PLANT DRY WEIGHT AT HARVEST (DAY 17) OF CUCUMBER SEEDLINGS GROWN IN VARIOUS STRENGTHS OF HOAGLAND'S SOLUTION[a]

| Strength of nutrient solution | Leaf Area ($cm^2$) | | Plant dry weight (g) | |
|---|---|---|---|---|
| | Changed | Not changed | Changed | Not changed |
| Full | 64.73 | 76.29 | 0.315 | 0.363 |
| $\frac{1}{2}$ | 67.60 | 58.81 | 0.322 | 0.331 |
| $\frac{1}{4}$ | 67.40 | 34.67 | 0.324 | 0.238 |
| $\frac{1}{8}$ | 55.09 | 18.06 | 0.306 | 0.165 |
| $\frac{1}{16}$ | 41.54 | 10.51 | 0.259 | 0.121 |
| $\frac{1}{32}$ | 22.51 | 5.39 | 0.184 | 0.073 |
| $\frac{1}{64}$ | 11.36 | 2.20 | 0.117 | 0.050 |
| $LSD_{0.05}$ | 12.34 | | 0.055 | |

[a] Nutrient solutions were changed every other day or not changed. In the latter case water was added every other day to compensate for transpiration. The interaction terms for concentration of nutrients and frequency of nutrient treatments were significant ($\alpha = 0.0001$).

ferulic acid on cucumber seedlings (data not presented) were not significantly different for the two nutrient treatments. Only the increasing ferulic acid (0, 0.5, 1, and 2 mM) concentrations significantly reduced dry weight and plant leaf area.

A model was developed to determine leaf area from the length and width of cucumber leaves. This eliminated the need for frequent destructive sampling. Length and width measurements of the leaves were converted to leaf area by the following equation:

$$\text{Seedling leaf area} = -1.457 + 0.00769(L \times W),\ \alpha = 0.0001,\ r^2 = 0.98,\ N = 121$$

where leaf area is in $cm^2$ and length ($L$) and width ($W$) values are in mm.

In a preliminary EM study, we had determined that cell number per unit area of 8- and 16-day-old cucumber leaves was increased by ferulic acid treatments. This observation suggested that ferulic acid decreased leaf cell expansion. We had made a similar observation for radicle growth of cucumber in a previous study (Blum et al., 1984). We also noted that ferulic acid treatments retarded root growth and development. In the presence of ferulic acid, roots were shorter, there were fewer secondary roots, and roots were tan to brown instead of white. The magnitude of the responses was concentration dependent.

The disappearance of ferulic acid (1 mM) from nutrient solution containing cucumber roots was constant beyond day 7 despite the fact that roots were placed into fresh ferulic acid solutions every other day. Between days 5 and 7, the ferulic acid concentration of the solution declined by 25%. Beyond day 7, the concentration of ferulic acid dropped by approximately 82% for each of the subsequent six 2-day periods. We also observed that microbial activity was at least partly responsible for the disappearance of ferulic acid since vanillic and protocatechuic acid were detected in solutions at change, and the cloudy nature of the solutions suggested the presence of microorganisms. The concentrations of vanillic and protocatechuic acid became progressively larger for each solution change. The maximum observed value was 10 ppm for vanillic acid and 2 ppm for protocatechuic acid. These two compounds have been identified as initial microbial metabolic products of ferulic acid (Turner and Rice, 1975; Blum et al., 1984). Thus an additional reason for changing nutrient solutions every other day was to minimize the population size of microbes in the nutrient solutions.

The fact that ferulic acid concentrations changed so rapidly also made it desirable to have a rapid bioassay procedure. Harvests of seedlings at short time intervals to determine rates of weight gains were not possible because of the constraints of our laboratory. Leaf area expansion appeared to be a good compromise since it did not require destructive sampling. In addition, leaf area of seedlings is frequently linearly correlated with seedling dry weight. This was the case in this study (see next section).

*Inhibition of Radicle Growth and Subsequent Seedling Growth.* Previously,

we had determined that radicle growth of cucumber was inhibited by ferulic acid (Blum et al., 1984). The question not resolved at that time was whether such reductions might affect subsequent seedling growth. As can be seen from the mean square values in Tables 2 and 3, treatments of seeds with ferulic acid during early stages of germination and radicle growth (up to 48 hr) did not significantly reduce growth of seedlings grown under either nutrient level. This suggested that the effects of ferulic acid on cucumber germination and radicle growth was simply a temporary delay in growth that was subsequently overcome. This suggestion is similar to that of Williams and Hoagland (1982) regarding seed germination (radicle emergence). They concluded that at least some phenolic acids simply delay the germination process.

Ferulic acid treatments beyond 48 hr, however, significantly reduced leaf area and dry weight of seedlings grown under both adequate and limiting nutrient levels (Table 2). There were also significant interactions between nutrient levels and ferulic acid treatments for seedling leaf area beyond day 15 and dry weights at final harvest. The former interactions appeared to be predominately due to the differences in the growth patterns of the seedlings in the two nutrient treatments since the patterns of inhibition were very similar (Figure 1). These findings for cucumber differ from those of Stowe and Osborn (1980) for barley. They noted that inhibitory effects of two phenolic acids (i.e., *p*-coumaric and vanillic acids) on barley were significant only at low nutrient concentrations. In addition in Figure 1 (A,B), note the similarity in pattern of seedling leaf area and plant dry weight for the various treatments at final harvest. The correlation coefficient for the full model (i.e., all treatments included, $r = 0.99$), the correlation coefficient after treatment effects were removed ($r = 0.96$) and the linear model for seedling leaf area and plant weight were highly significant (leaf area in $cm^2 = -25.6 + 260.7$ (plant weight in g), $\alpha = 0.0001$, $r^2 = 0.94$, $N = 54$).

Figure 2 presents the mean absolute rates of leaf expansion ($cm^2$/seedling/2 days) for these seedlings. Both the nutrient and the ferulic acid treatments were significant for all but the first growth period. The interaction terms for nutrient and ferulic acid treatments were significant for the last four growth periods. Nutrition became a major limiting factor for growth of the seedlings in the $\frac{1}{16}$-strength solution at some time beyond day 13. Percent reductions of the mean absolute rate associated with ferulic acid treatments, however, were very similar. For example, reductions for growth period 15–17, the period during which maximum percent reduction occurred, were 52% and 72% for the 0.5 and 1.0 mM ferulic acid treatments given to seedlings grown in full-strength solutions and 58% and 80% for seedlings grown in the $\frac{1}{16}$-strength solutions.

Figure 2 also presents the data for mean relative rates of leaf expansion. Mean relative rates of leaf expansion ($cm^2/cm^2$/2 days) of cucumber seedlings initially increased and then decreased. These broad features of change are an expression of ontogenetic drift which are associated with normal development.

TABLE 2. SUMMARY OF MEAN SQUARE VALUES FROM ANALYSIS OF VARIANCE FOR LEAF AREA OF CUCUMBER SEEDLINGS TREATED WITH FERULIC ACID AND GROWN UNDER TWO NUTRIENT LEVELS[a]

| | | Plant age (days from seed) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *df* | 9 | 11 | 13 | 15 | 17 | 19 | 21 |
| PSEED[b] | 2 | 0.066 | 1.636 | 1.207 | 19.685 | 58.600 | 44.950 | 94.135 |
| SEEDL | 2 | 0.182[c] | 128.855[c] | 794.063[c] | 2868.217[c] | 9381.470[c] | 21790.475[c] | 35416.366[c] |
| NUT | 1 | 0.409[c] | 83.570[c] | 515.725[c] | 2435.396[c] | 11965.775[c] | 45032.968[c] | 104192.609[c] |
| PSEED + SEEDL | 4 | 0.023 | 1.3977 | 7.803 | 18.975 | 14.499 | 57.489 | 127.103 |
| PSEED + NUT | 2 | 0.088 | 8.577 | 21.450 | 64.005 | 159.419 | 203.894 | 331.200 |
| SEEDL + NUT | 2 | 0.102 | 4.649 | 15.710 | 126.225[c] | 910.244[c] | 3448.089[c] | 7487.594[c] |
| PSEED + SEEDL + NUT | 4 | 0.024 | 0.261 | 1.672 | 6.438 | 3.611 | 33.763 | 95.019 |
| Error | 36 | 0.064 | 7.008 | 17.473 | 41.841 | 103.431 | 263.138 | 424.981 |

[a] Phenolic acid treatments: 0, 0.5, and 1.0 mM; solutions were changed every other day. Nutrient levels: full strength and $\frac{1}{6}$ strength Hoagland's solution.
[b] PSEED = phenolic acid treatments of seeds, SEEDL = phenolic acid treatments of seedlings, and NUT = nutrient treatments.
[c] Significance level: $\leq 0.05$ level of probability.

TABLE 3. SUMMARY OF MEAN SQUARE VALUES FROM ANALYSIS OF VARIANCE FOR SHOOT, ROOT, AND PLANT DRY WEIGHT OF 21-DAY-OLD CUCUMBER SEEDLINGS TREATED WITH FERULIC ACID AND GROWN UNDER TWO NUTRIENT LEVELS[a]

| | *df* | Shoot | Root | Plant |
|---|---|---|---|---|
| PSEED[b] | 2 | 0.0005 | 0.0005 | 0.0018 |
| SEEDL | 2 | 0.4945[c] | 0.0257[c] | 0.7456[c] |
| NUT | 1 | 0.7601[c] | 0.0087[c] | 0.9310[c] |
| PSEED + SEEDL | 4 | 0.0011 | 0.0004 | 0.0028 |
| PSEED + NUT | 2 | 0.0054 | 0.0007 | 0.0098 |
| SEEDL + NUT | 2 | 0.0506[c] | 0.0005 | 0.0599[c] |
| PSEED + SEEDL + NUT | 4 | 0.00058 | 0.0001 | 0.0009 |
| Error | 36 | 0.0050 | 0.0004 | 0.0081 |

[a] Phenolic acid treatments: 0, 0.5, and 1.0 mM; solutions were changed every other day. Nutrient levels: full-strength and $\frac{1}{16}$-strength Hoagland's solution.
[b] PSEED = phenolic acid treatments of seed, SEEDL = phenolic acid treatments of seedlings, and NUT = nutrient treatments.
[c] Significance level: $\leq 0.05$ level of probability.

Although mean absolute leaf expansion is informative, in reality it is a poor comparative character when one remembers the differences in leaf area for the various treatments with time. Mean relative leaf expansion is much more informative when comparisons are to be made, since it expresses growth on a per unit area basis instead of a leaf or a plant basis. The interaction terms for nutrient and ferulic acid treatments were no longer significant except for the 13- to 15-day growth period. For all growth periods but the 13- to 15-day, the main treatment effects of nutrient and ferulic acid were significant. What was surprising was that the mean relative rate of leaf expansion recovered so rapidly even with the continued application of ferulic acid. Recall that ferulic acid solutions were changed every other day. Recovery was more rapid under the full- than the $\frac{1}{16}$-strength nutrient treatments.

*Recovery after Treatment.* Recovery of growth occurred rapidly once seedlings were removed from ferulic acid treatments (Figures 3 and 4). The mean absolute rate of leaf expansion (Figure 3) was partially suppressed as long as ferulic acid was supplied. This was similar to that observed in the previous experiment. However, once the ferulic acid was removed from the root environment, the plant's mean absolute rate increased rapidly.

Mean relative rates of leaf expansion (Figure 4) also recovered rapidly. All treatment rates were eventually equal to or greater than the control expansion rates. Behavior of the first and second leaf were similar in this regard. These data suggest that the initial application of ferulic acid was a "shock" to the rate of growth that was overcome with time. The amount of time required for recov-

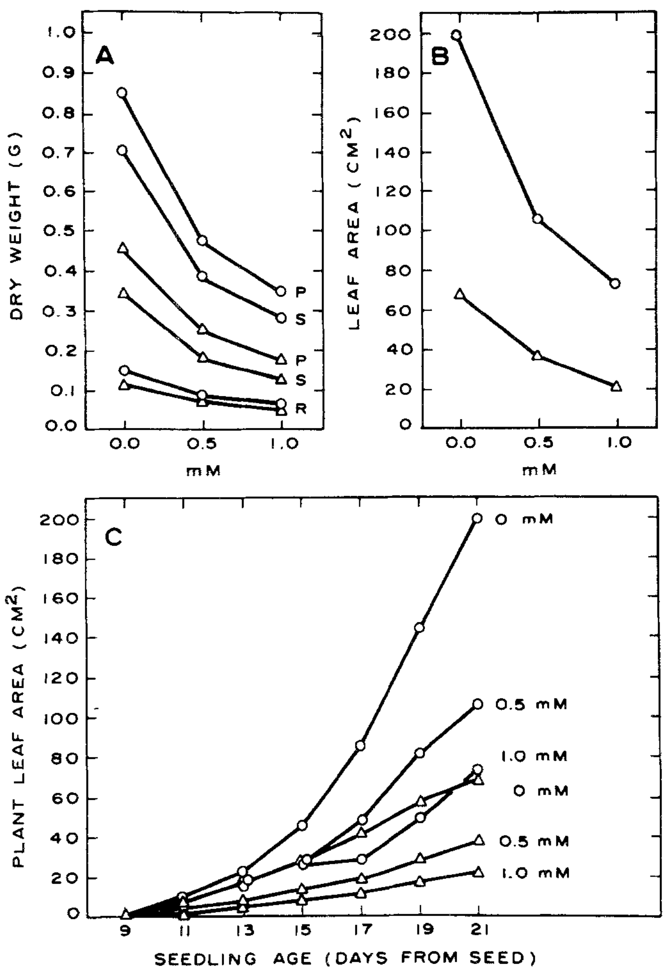


FIG. 1. Effects of multiple treatments of various concentrations of ferulic acid in full (o) and $\frac{1}{16}$ ($\Delta$) strength Hoagland's solutions on cucumber seedling dry weight (A) and leaf area (B, C). (A) P = plant, S = shoot, and R = root. (A) and (B) are based on final harvest data. Solutions were changed every other day. Plants were harvested when 21 days old ($N$ = 9, means include values for pretreatment of seeds).

ery was related to the initial concentration of ferulic acid. The "shock" and subsequent delay in recovery, however, were significant enough, so that within the time span of this experiment, significant differences were observed for both the total plant leaf area and plant dry weight (data not presented). Also note the difference in mean relative rates of leaf expansion for growth period 7–9 in Figure 4 and Figure 2. Since germination and growth of cucumber are so rapid,

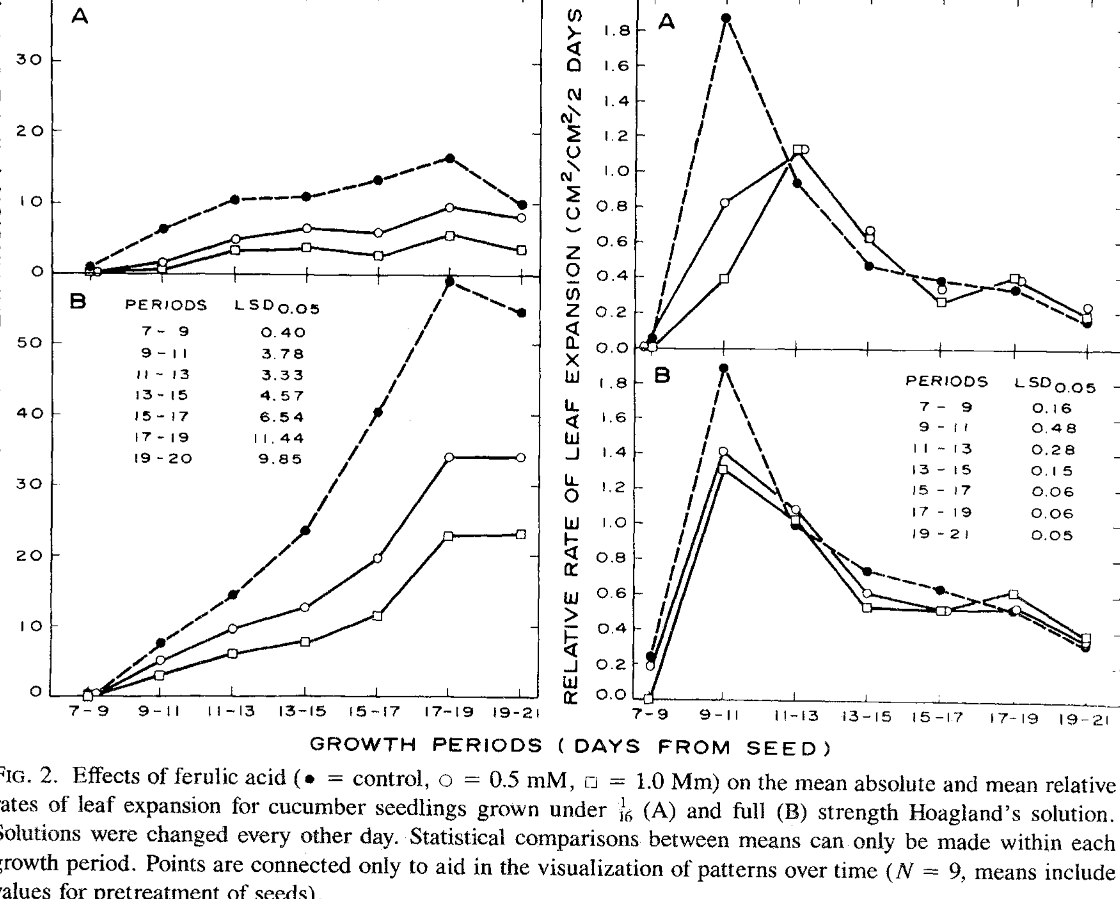


FIG. 2. Effects of ferulic acid (• = control, ○ = 0.5 mM, □ = 1.0 Mm) on the mean absolute and mean relative rates of leaf expansion for cucumber seedlings grown under $\frac{1}{16}$ (A) and full (B) strength Hoagland's solution. Solutions were changed every other day. Statistical comparisons between means can only be made within each growth period. Points are connected only to aid in the visualization of patterns over time ($N = 9$, means include values for pretreatment of seeds).

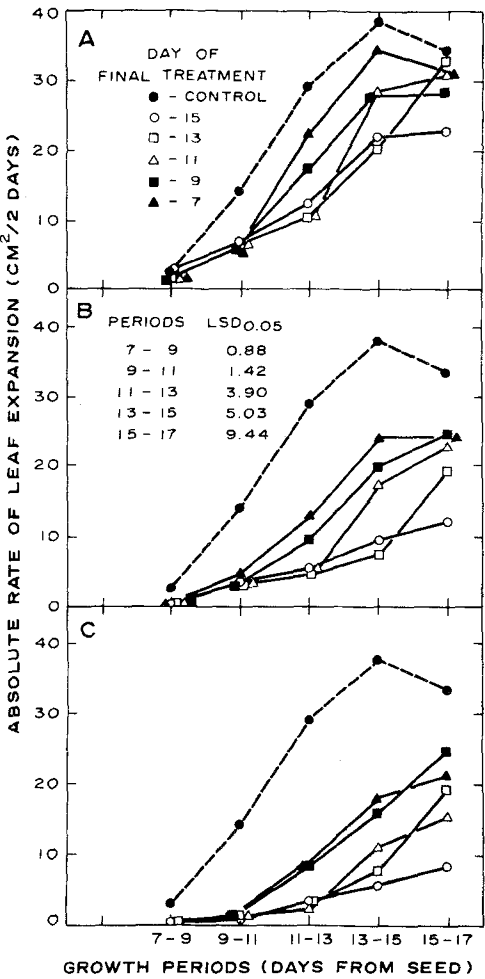


FIG. 3. Effects of ferulic acid (A = 0.5 mM, B = 1.0 mM, C = 2.0 mM) in full-strength Hoagland's solution on mean absolute rates of leaf expansion of cucumber seedlings. Solutions were changed every other day. All ferulic acid treatments were initiated when seedlings were 7 days old. Subsequently, sets of seedlings were removed from ferulic acid treatments at 2-day intervals. Statistical comparisons between means can be made only within each growth period. Points are connected only to aid in the visualization of the recovery of absolute rates ($N = 3$).

slight differences in temperature, for example, will make a substantial difference in the stage of development for a given point in time.

*Time of Initial Treatment.* Percent inhibition was little modified with seedling age (Figure 5). The absolute rate of leaf expansion was reduced roughly 58, 89, and 96% for 0.5, 1.0, and 2.0 mM ferulic acid, respectively. Plants

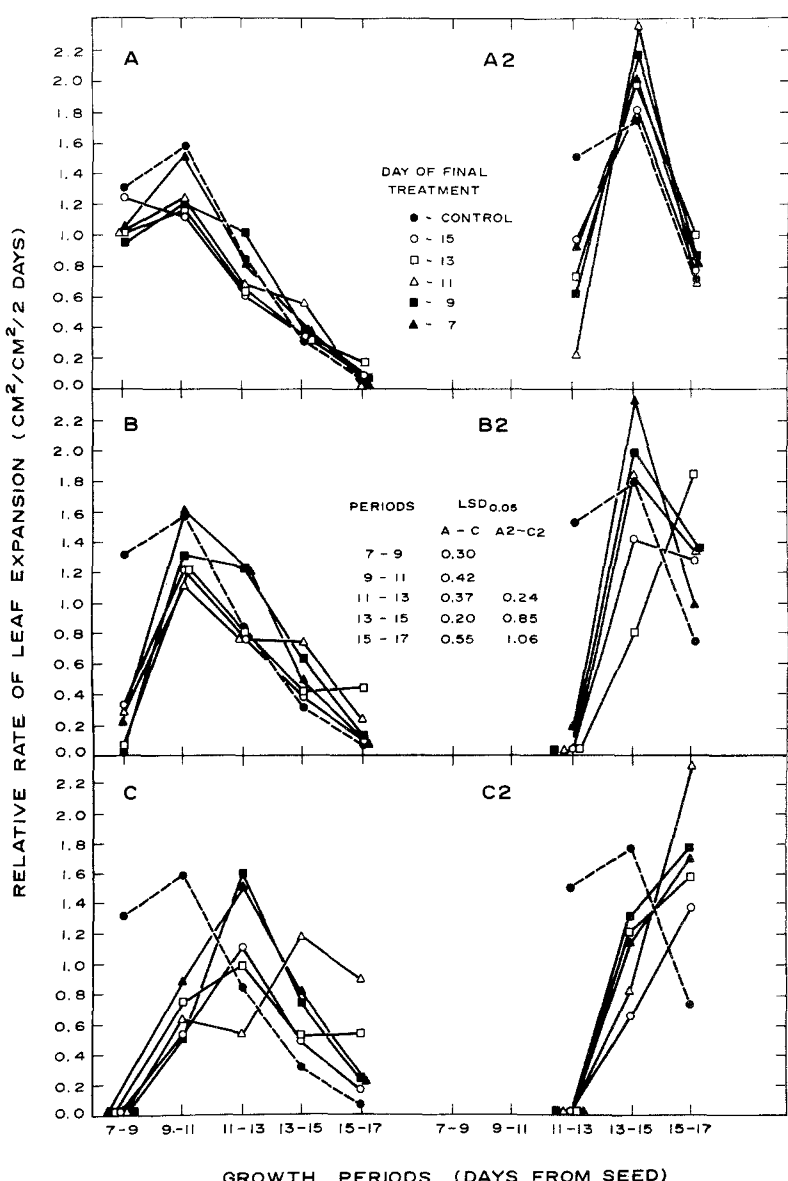


FIG. 4. Effects of ferulic acid (A = 0.5 mM, B = 1.0 mM, C = 2.0 mM) on mean relative rates of leaf expansion for the first leaf (A–C) and the second leaf (A2–C2). For additional detail, see Figure 3.

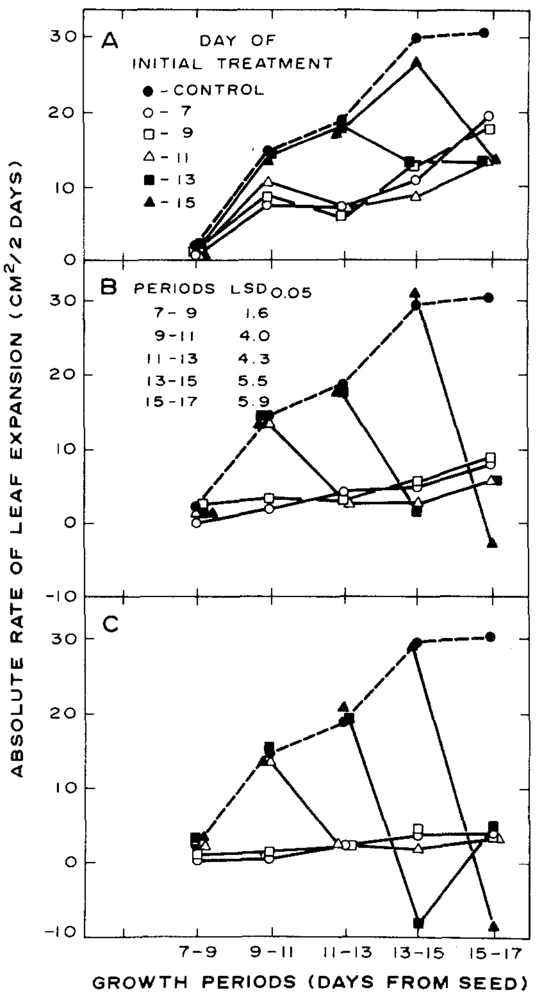


FIG. 5. Effects of ferulic acid (A = 0.5 mM, B = 1.0 mM, C = 2.0 mM) in full-strength Hoagland's solution on mean absolute rates of leaf expansion of cucumber seedlings. Solutions were changed every other day. Initial ferulic acid treatments varied, but all seedlings were treated during the last growth period. Seedlings were grown in Hoagland's solution until ferulic acid treatment was initiated. Statistical comparisons can only be made within each growth period. Points are connected only to aid in the visualization of the initial impact of ferulic acid ($N = 3$).

wilted within a few hours (i.e., negative mean absolute growth rates) after the initial 1 or 2 mM ferulic acid application for growth periods 13–15 and 15–17. However, plants usually recovered completely within 48 hr (i.e., leaves were turgid). Thus the initial "shock" was even more evident here.

Wilting, of course, indicates a negative net water balance for a leaf. To test

whether the observed wilting may have been due to the water potentials of the ferulic acid solutions, we grew cucumber plants in solutions of roughly equivalent osmolalities. Initial milliosmoles (mOsm) of the solutes at day 14, when initial treatments were applied, were 22 for Hoagland's solution, 33 for the 2 mM ferulic acid in nutrient solution, and 27 for polyethylene glycol (PEG, 6000 mol wt) in nutrient solution. Solutions were changed on day 16. Leaf expansion was completely inhibited by ferulic acid. Leaf area produced in the presence of PEG was not significantly different from the leaf area of plants grown in Hoagland's solution. Leaves of plants treated the first time, but not the second time, with ferulic acid wilted within 1.5 hr after treatment. Leaves were turgid again, however, within 48 hr. Water utilization expressed as ml/cm$^2$ leaf area/2 days were as follows over days 14–16 and 16–18: Hoagland's solution, 0.45 and 0.45; PEG–nutrient solution, 0.40 and 0.43; and ferulic acid–nutrient solution, 0.07 and 0.1, respectively. Note that water utilization was reduced even after wilted leaves had recovered their turgidity. This suggested that the stomata were closed at that point.

Einhellig and Kuan (1971) noted that scopoletin and chlorogenic acid at millimolar concentrations caused stomatal closure in tobacco and sunflower seedlings within 24 hr after treatment. This closure persisted for several days. Differential utilization of water as noted would modify the milliosmoles of the solutes in the bottles over time. The milliosmoles on day 16 (lowest water level in bottle) were as follows: 31 for Hoagland's solution, 35 for the PEG–nutrient solution, and 29 for the ferulic acid–nutrient solution. The increase in milliosmoles of the solutes for the Hoagland's solution and the PEG solution was related to the loss of water from the bottles by transpiration. The decline in milliosmoles of the solutes in the ferulic acid solution, we assume, was related to the loss of ferulic acid from the solution and the small loss of water from the bottles by transpiration. Based on these data, we concluded that the impact of ferulic acid on leaf expansion of cucumber plants could not be explained by the differences in water potential of the solutions.

In the previous experiment, we noted rapid recovery for mean relative rates of leaf expansion when plants were continuously treated (i.e., every other day) with ferulic acid (Figure 4). This can also be seen for seedlings treated initially at the 7–9 growth period (Figure 6), but note that the magnitude of recovery declined considerably as seedlings were initially treated at later ages. Since, with leaf age, the potential for recovery declines (i.e., mean relative growth rates decline with leaf age, reaching zero at full expansion), the proportion of the various leaf stages present (i.e., actively expanding vs. nonexpanding) for a seedling during ferulic acid treatment would appear to be important here.

*Acclimation to Ferulic Acid.* No statistically significant differences were observed for plant responses to 1 mM ferulic acid when plants were grown in 0, 0.1, or 0.2 mM ferulic acid. Thus seedlings were not acclimated by the pre-

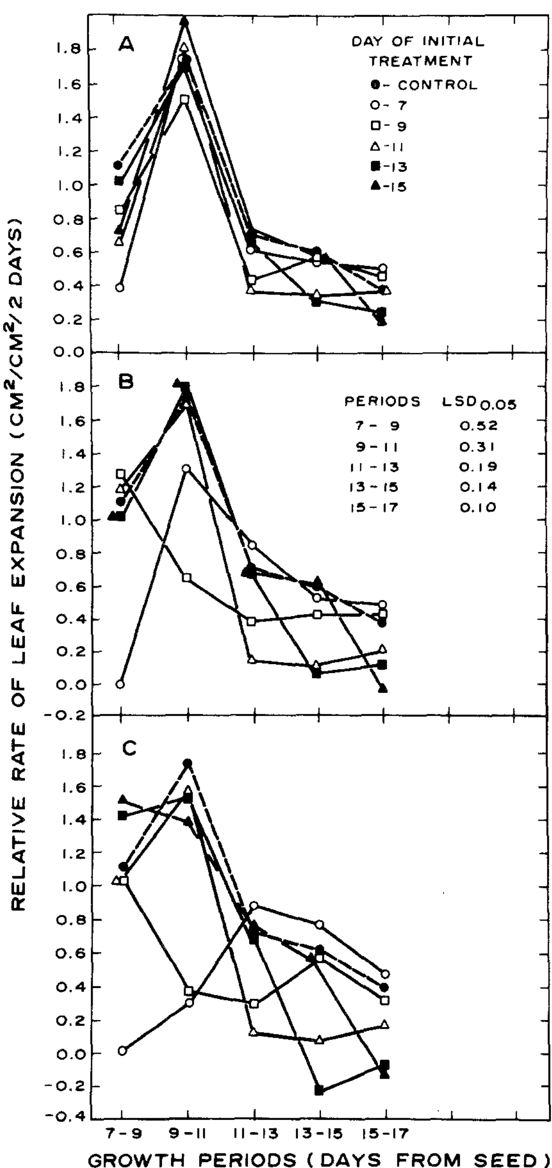


FIG. 6. Effects of ferulic acid (A = 0.5 mM, B = 1.0 mM, C = 2.0 mM) in full-strength Hoagland's solution on mean relative rates of leaf expansion of cucumber seedlings. For additional details, see Figure 5.

TABLE 4. EFFECTS OF SINGLE TREATMENT OF FERULIC ACID GIVEN AT VARIOUS AGES ON DRY WEIGHT AND LEAF AREA OF SEEDLINGS AT FINAL HARVEST[a]

| | | | Time of treatment (days from seed) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | $LSD_{0.05}$ | Control | 7 | 9 | 11 | 13 | 15 | 17 | 19 |
| Dry weight (g) | | | | | | | | | |
| Shoot | 0.056 | 0.564 | 0.459 | 0.458 | 0.447 | 0.408 | 0.380 | 0.380 | 0.430 |
| Root | 0.016 | 0.123 | 0.095 | 0.102 | 0.102 | 0.095 | 0.087 | 0.085 | 0.097 |
| Plant | 0.071 | 0.687 | 0.554 | 0.559 | 0.549 | 0.503 | 0.467 | 0.465 | 0.527 |
| Leaf area ($cm^2$) | | | | | | | | | |
| Plant | 18 | 181 | 140 | 138 | 129 | 121 | 107 | 100 | 125 |
| Leaf 1 | 6 | 54 | 40 | 40 | 40 | 39 | 41 | 41 | 48 |
| Leaf 2 | 7 | 74 | 67 | 67 | 61 | 59 | 51 | 42 | 56 |
| Leaf 3 | 9 | 46 | 30 | 28 | 25 | 21 | 14 | 16 | 19 |
| Leaf 4 | 3 | 7 | 3 | 3 | 3 | 2 | 1 | 1 | 2 |

[a] Seedlings were treated with a 1 mM ferulic acid–nutrient solution for two days starting with the day given in the table. At other times seedlings were grown in full-strength Hoagland's solution containing 0, 0.1, or 0.2 mM ferulic acid which was changed every other day ($N = 9$).

treatment of noninhibitory levels of ferulic acid. There were, however, significant time of treatment effects for leaf area (Table 4) and mean absolute and mean relative rates of leaf expansion (Figures 7 and 8) associated with the 1 mM ferulic acid treatments. The initial drop in leaf expansion, as well as recovery, was evident in both cases. Individual leaves, when present and actively expanding, responded in a similar manner.

At harvest, dry weights and leaf areas were reduced by the 1 mM ferulic acid treatments (Table 4). Reductions in plant dry weight and plant leaf area ranged from 19% to 32% and from 25% to 45%, respectively. Effects of even the earliest 1 mM ferulic acid treatment (7–9 growth period) resulted in a significant reduction of leaf area and dry weight at final harvest (day 21).

## SUMMARY AND CONCLUSIONS

The disappearance of ferulic acid from the nutrient cultures of cucumber seedlings was expected, since we had noted in an earlier study a similar disappearance of ferulic acid from solutions in Petri dishes containing germinating cucumber seeds (Blum et al., 1984). In both instances, we noted a production of vanillic and protocatechuic acid in the solutions. These have been identified as initial products of microbial metabolism of ferulic acid (Evans, 1963; Flaig, 1964; Dagley, 1971; Turner and Rice, 1975; Martin and Haider, 1976). Since the seeds were not sterilized, it was not possible to determine the actual cause of the decline of ferulic acid in the nutrient cultures. But we suspect that it was

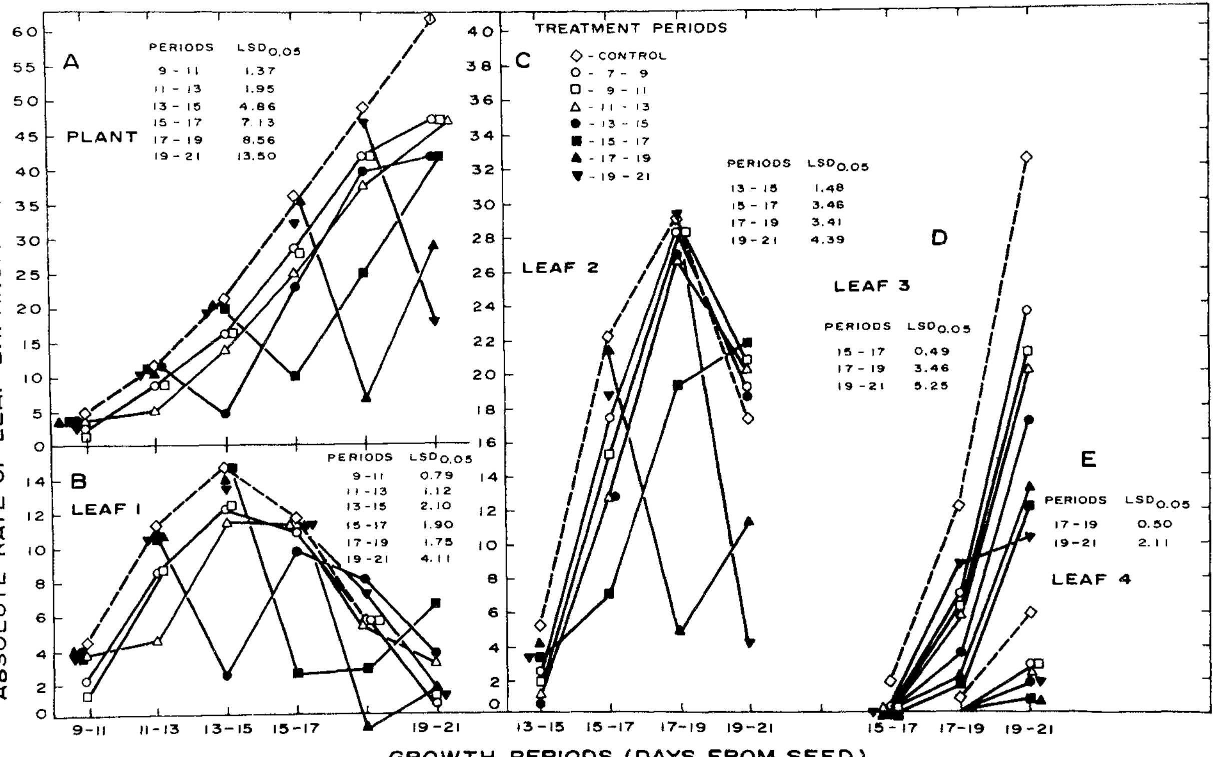


FIG. 7. Effects of a single 2-day 1 mM ferulic acid treatment on mean absolute rates of leaf expansion of seedlings of various ages and individual leaves of seedlings grown in full-strength Hoagland's solution. (A) describes values for seedlings, (B) for leaf 1, (C) for leaf 2, (D) for leaf 3, and (E) for leaf 4. Statistical comparisons can only be made within each growth period. Points are connected only to aid in the visualization of the impact of ferulic acid ($N = 9$, means include values for noninhibitory levels of ferulic acid).

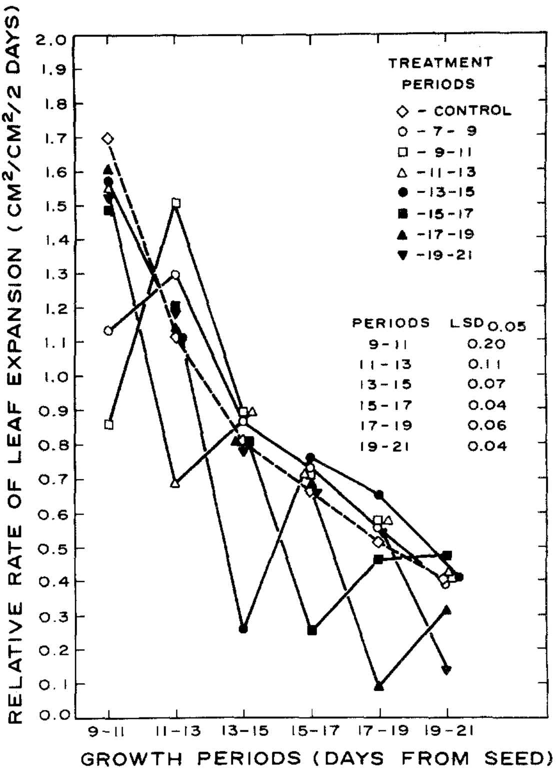


FIG. 8. Effects of a single 2-day 1 mM ferulic acid treatment on mean relative rates of leaf expansion of seedlings of various ages. For additional details, see Figure 7.

a combination of microbial activity, physical degradation, and root adsorption and/or absorption. Since the disappearance of ferulic acid occurred so rapidly (i.e., less than 48 hr for 14- to 16-day-old seedlings), it was not practical to maintain a constant concentration of ferulic acid in the solutions. We chose therefore, in some instances, to change the solutions every other day. Thus, for a number of our experiments, the ferulic acid concentrations were brought back to their original concentrations every other day. In other experiments only a single treatment was employed.

The effects of ferulic acid on leaf area expansion of plants that did not wilt could be detected within less than 48 hr after the initial treatment. Our data suggest that the reduction in leaf expansion associated with ferulic acid treatments was at least in part a result of modified water utilization. Thus, the observed modification of leaf area expansion appears to be a secondary effect of ferulic acid. The rapid recovery of leaf expansion after the seedlings were removed from the ferulic acid solutions also tends to support this conclusion.

Whether the ultimate impact of ferulic acid on dry matter production was due to this induced water imbalance or some other mechanisms cannot be stated.

Patterson (1981), for example, noted that ferulic acid reduced soybean photosynthesis rates, stomatal conductance, and leaf water potentials in a similar manner. He also noted a loss of chlorophyll with time after ferulic acid treatment. A decline of chlorophyll content of soybean leaves was also observed after ferulic acid treatment by Einhellig and Rasmussen (1979). Einhellig and Kuan (1971) suggested that reductions in stomatal aperature induced by millimolar concentrations of scopoletin were correlated with photosynthetic reductions in tobacco and sunflower. Harper and Balke (1981) noted that ferulic acid inhibited the uptake of potassium by oat roots. How such modifications of potassium uptake by roots may be related to water uptake is not known. We also observed that the recovery rates of cucumber seedlings were slower in nutrient-limited solutions (i.e., $\frac{1}{16}$ vs. full strength). This may be a result of reduced metabolism of cucumber seedlings in nutrient-limiting environments. It appears, therefore, that ferulic acid brings about a reduction in leaf expansion that is reversible in actively expanding leaves. However, even a short-term reduction in leaf expansion for rapidly growing cucumber seedlings results in a significant loss in seedling dry weight in comparison to control plants. These reductions occurred both in adequate and limiting nutrient environments.

The magnitude of the reduction in leaf expansion was related to the concentration of ferulic acid in the solution. Data presented here indicate that the modification of water uptake by the roots was not due to the differences in water potential of the various solutions.

Ferulic acid-induced reductions of leaf expansion could only be maintained by continued treatments of ferulic acid. Even here there was some recovery. The magnitude of recovery depended on the concentration of ferulic acid, frequency of ferulic acid application, plant nutrition, the age of the seedling and, we suspect, the rates of microbial metabolism of ferulic acid.

In soil environments, low, noninhibitory levels of ferulic acid are no doubt present at various times. This could lead to "hardening" (i.e., acclimation) of seedlings to ferulic acid. Cucumber seedlings allowed to develop in the presence of noninhibitory concentrations of ferulic acid were not significantly modified in their sensitivity to subsequent inhibitory levels of ferulic acid. Thus, at least over the period of this study, cucumber seedlings were not "hardened" by low-level ferulic acid treatments.

For the given conditions of these experiments, we may conclude: (1) that the inhibition of radicle growth of cucumber by a single ferulic acid treatment is a short-term inhibition that has little effect on subsequent seedling growth in nutrient culture: (2) that the inhibition of cucumber leaf area expansion by ferulic acid is a result of an initial "shock" that appears to be a result of an induced water imbalance; (3) that the reduction in leaf area is due to the delay in leaf area expansion associated with the initial "shock"; (4) that the magni-

tude of recovery of leaf area expansion depends on the number of ferulic acid treatments, the concentration of ferulic acid, nutrient status of the seedling and the stage of development of a particular leaf; (5) that leaf area expansion is a useful character that may be used to monitor the effects of ferulic acid on cucumber growth and development; and (6) that it appears that noninhibitory levels of ferulic acid in the root environment do not "harden" cucumber seedlings to subsequent inhibitory levels of ferulic acid.

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# MASS SPECTRAL DETERMINATION OF ALDEHYDES, KETONES, AND CARBOXYLIC ACIDS USING 1,1-DIMETHYLHYDRAZINE[1]

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**Abstract**—Analyses of nanogram to milligram quantities of aliphatic aldehydes, fatty acids, and unhindered aliphatic ketones such as those typically found in pheromonal blends have been effected by treating these mixtures with 1,1-dimethylhydrazine. The aldehydes and ketones form *N,N*-dimethylhydrazones, while the fatty acids form methyl esters. Structural elucidation of the reaction products was achieved using EI and CI gas chromatography–mass spectrometry.

**Key Words**—Semiochemicals, pheromones, methyl esters, *N,N*-dimethylhydrazones, *N,N*-dimethylhydrazine, mass spectrometry.

## INTRODUCTION

Insects produce a variety of semiochemicals that govern many, if not most, of their behavioral activities. Such chemicals are typically produced in nanogram to microgram quantities as complex mixtures of functionally diverse components (Shorey and McKelvey, Jr., 1977; Ritter, 1979). Common to many of these semiochemical blends are aldehydes, ketones, and carboxylic acids (Brand et al., 1979; Silverstein, 1977; Tamaki, 1977). The interpretation of the mass spectra of these aliphatic aldehydes and ketones is not always straightforward,

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however. These compounds frequently possess low abundance or nonexistent molecular ions. Aldehydes can have prominent M-18 ions, making it difficult to distinguish them from unsaturated alcohols, and, of course, diunsaturated alcohols are not easily differentiated from monounsaturated aldehydes by either electron impact (EI) or chemical ionization (CI) mass spectrometry.

In the course of our investigation of the marking pheromone complexes of various *Philanthus* spp. (McDaniel et al., unpublished work), we encountered complex mixtures of aldehydes, ketones, and free acids. We therefore sought a derivatization technique that would not only allow chromatographic separation, but would also allow unequivocal mass spectral characterization of the products.

Reaction of a carbonyl moiety with 1,1-dimethylhydrazine (DMH) to form the *N,N*-dimethylhydrazone as synthetic intermediate has been previously reported (Smith and Walker, 1962; Newkome and Fishel, 1966; Corey and Enders, 1976). VandenHeuvel and Horning (1963) have used the hydrazone products of ketones for gas chromatographic analyses, relying solely on retention times for characterization. GC-MS analysis is a much more powerful technique that potentially offers a facile, unequivocal characterization of *N,N*-dimethylhydrazones derived from aldehydes and ketones.

We found that *N,N*-dimethylhydrazone formation proceeded smoothly and essentially quantitatively for aliphatic aldehydes. Good yields were obtained for aliphatic unhindered ketones, whereas hindered ketones produced only modest yields. We discuss the mass spectra of these derivatives and their application to structural elucidation of the parent carbonyl compound.

The DMH reagent surprisingly converted similar quantities of aliphatic carboxylic acids to their corresponding methyl esters. We were thus able, after the addition of a single reagent, to perform GC-MS analyses of mixtures of aldehydes, ketones, and carboxylic acids.

## METHODS AND MATERIALS

Hydrazone formation was achieved by the addition of approximately 1 mmol of neat 1,1-dimethylhydrazine (Mallinckrodt, St. Louis, Missouri) to approximately $10^{-4}$–$10^{-2}$ mmol of the aldehyde or ketone. Reactions were carried out in Wheaton 5-ml Reactivials® sealed with Teflon®-lined caps. The samples were held at 50° for approximately 1 hr in a Pierce Reacti-Therm®. Appropriate dilutions were made prior to analysis by the addition of *n*-hexane. Yields were estimated using FID gas chromatography by comparing peak areas of the starting material to those of the hydrazone product.

Esterification of fatty acids was examined using stearic acid as a model compound. One millimole stearic acid was dissolved in 100 ml THF; 100-$\mu$l aliquots (1.0 $\mu$mol stearic acid) were placed in the Reactivials, the solvent removed with $N_2$, and 100 $\mu$l (1.3 mmol) neat 1,1-dimethylhydrazine was added.

The vials were sealed with Teflon-lined caps and allowed to react for 1 hr at 50°C. The 1,1-dimethylhydrazine was removed with a stream of nitrogen, and an appropriate volume of THF was added. Conversion was quantitated by gas chromatographic comparison to standard solutions of methyl stearate.

Gas chromatographic analyses were performed on a Hewlett-Packard 5710 gas chromatograph with a FID detector. The column employed was a 1.8-m × 2-mm ID glass column packed with 3% SP-2100 on 100/120 mesh Supelcoport.

Gas chromatographic–mass spectrometric (GC-MS) analyses were performed on a Hewlett-Packard 5710A gas chromatograph–5982 mass spectrometer interfaced to a 5933 data system. Compounds were separated on a glass 2-m × 2-mm ID column packed with 3% SP-2100 on 100/120 mesh Supelcoport. Electron impact spectra were generated with a source potential of 70 eV; chemical ionization spectra were generated using ultrapure methane (Airco, Incorporated) as both the carrier gas and the ionizing gas. The internal source pressure was 0.5 torr, with a source potential of 200 eV.

## RESULTS AND DISCUSSION

While the aldehydes examined reacted essentially quantitatively with DMH under our experimental conditions, the reactivity of DMH with ketones varied dramatically, with yields appearing to be quite susceptible to steric hindrance. This observation is in agreement with previous reports (VandenHeuvel and Horning, 1963). Hydrazone yields are presented in Table 1.

The EI mass spectra of the *N,N*-dimethylhydrazones of the aldehydes were very definitive. All had a McLafferty rearrangement ion ($m/z = 86$) as the base

TABLE 1. CONVERSION OF ALDEHYDES AND KETONES TO THEIR *N,N*-DIMETHYLHYDRAZONES

| Parent compound | Conversion (%) |
|---|---|
| 11-Undecenal | 100 |
| Tetradecanal | 100 |
| *Z*-9-Tetradecenal | 95 |
| *Z*-11-Hexadecenal | 100 |
| 2-Heptanone | 61 |
| 3-Heptanone | 86 |
| 4-Heptanone | 15 |
| 4-Octanone | 17 |
| 2-Methyl-4-octanone | 2 |
| 3-Methyl-4-octanone | 3 |
| 2,6-Dimethyl-4-heptanone | 2 |
| 2-Undecanone | 58 |

TABLE 2. MASS SPECTRAL DATA OF *N,N*-DIMETHYLHYDRAZONES OF SELECTED ALDEHYDES

| | Diagnostic ions: $m/z$ and (abundances) | |
|---|---|---|
| Parent compound | M+ | Base peak |
| 11-Undecenal | 210 (33%) | 86 |
| Tetradecanal | 254 (22%) | 86 |
| *Z*-9-Tetradecenal | 252 (23%) | 86 |
| *Z*-11-Hexadecenal | 280 (21%) | 86 |
| *E*-14-Methyl-8-hexadecenal | 294 (31%) | 86 |
| 12-Oxoheptacosanal | 464 (16%) | 86 |

peak, with molecular ions of typically 20–30% relative abundance (Table 2). Chemical ionization mass spectra of these hydrazones had the $(M+1)^+$ ions as their base peaks with little other structural information. Typical EI and CI spectra are presented in Figure 1.

The EI mass spectral data of the *N,N*-dimethylhydrazones of some representative ketones are presented in Table 3. Usually, the base peak for these hydrazones was at $m/z$ = 44, the dimethylamino cation. For the hydrazone of 2-ketones, the McLafferty ion at $m/z$ = 100 was always prominent and was the base peak for the hydrazone of 2-pentadecanone. When the derivatized keto functionality was more internally positioned on unbranched ketones, less prominent but still diagnostically useful rearrangement ions were present. Other types of ketone hydrazones yielded either few or no McLafferty rearrangement ions. In these cases, preparation of the *N,N*-dimethylhydrazone provided little additional useful structural information. CI mass spectra of the ketone hydrazones, as in the case of the aldehyde hydrazones, had the $(M+1)^+$ ion as the base peak, and possessed no other ions which were diagnostically useful. Representative EI and CI mass spectra for the *N,N*-dimethylhydrazone of 2-pentadecanone are shown in Figure 2.

During our investigation of the reaction of DMH with aldehydes, we found that the aldehydes contained significant amounts of corresponding carboxylic acids. Unexpectedly, the addition of DMH to these aldehyde–carboxylic acid mixtures resulted not only in the expected hydrazone formation, but also in the formation of the methyl esters of the carboxylic acids. We examined this reaction using micromole quantities of stearic acid and obtained a conversion of 62 ± 9% ($\bar{X}$ ± SD) for eight replications. Earlier runs with nanomolar quantities of acid and millimolar quantities of DMH were essentially quantitative, presumably because of mass action.

The mass spectra of methyl esters are well known and will not be discussed (Ryhage and Stehagen, 1963; Heller and Milne, 1978).

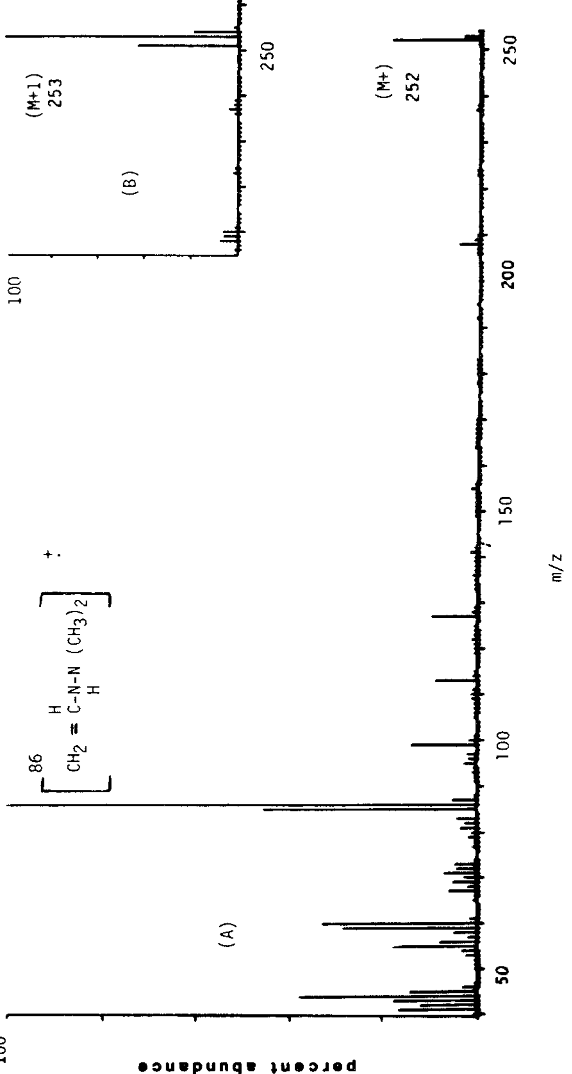


FIG. 1. Mass spectra of the *N,N*-dimethylhydrazone of Z-9-tetradecenal: (A) EI; (B) CI.

TABLE 3. DIAGNOSTIC MASS SPECTRAL FRAGMENT IONS OF *N,N*-DIMETHYLHYDRAZONES OF SELECTED KETONES

| | Diagnostic ions, *N,N*-dimethylhydrazones: *m/z* (relative abundance) | | |
|---|---|---|---|
| Parent compound | M+ | Base peak | McLafferty rearrangement |
| 2-Methyl-2-hexen-4-one | 154 (11%) | 44 | --- |
| 3-Heptanone | 156 (73%) | 44 | 144 (25%) |
| 2-Octanone | 170 (45%) | 44 | 128 (14%), 142 (0.2%) |
| 2-Methyl-3-heptanone | 170 (20%) | 44 | 128 (2%) |
| 4-Methyl-3-heptanone | 170 (8%) | 44 | 128 (2%) |
| 2-Methyl-4-heptanone | 170 (62%) | 44 | 128 (0%), 142 (0%) |
| 3-Methyl-4-heptanone | 170 (25%) | 44 | 142 (0%) |
| 2,6-Dimethyl-4-heptanone | 184 (15%) | 44 | 142 (0%) |
| 2-Methyl-4-octanone | 184 (39%) | 44 | 142 (1%) |
| 2-Undecanone | 212 (9%) | 44 | 100 (67%) |
| 2-Pentadecanone | 268 (11%) | 100 | 100 (100%) |

The mechanism of the esterification reaction has not yet been established, but is currently being investigated. One possibility is that esterification of the carboxylic acids might be occurring in the injection port of the gas chromatograph via a thermolysis of the acid–DMH salt:

$$R-C(=O)O^{-} \;\; {}^{+}NH_3N(CH_3)_2 \rightarrow R-C(=O)-O-CH_3$$

To examine this possibility, we removed excess DMH from the reaction mixture and partitioned the nonvolatile residue between hexane and 0.1 N $H_2SO_4$. The stronger mineral acid should release the weaker carboxylic acid from the salt form if it is present. Subsequent gas chromatographic and GC-MS analysis of the hexane layer showed no diminution of the amount of ester product. This hexane layer was also examined by infrared spectrophotometry. The IR spectrum clearly established that esterification was occurring in the DMH solution.

The use of 1,1-dimethylhydrazine to characterize aldehydes, simple alkyl ketones, and fatty acids thus appears to offer great potential in the analysis of insect semiochemicals. The method will frequently obviate the necessity to preparatively isolate individual components and will allow structural analysis of quantities of semiochemicals approximating that in a single insect. We are currently using this reagent in our laboratory in just such a manner for pheromone analyses.

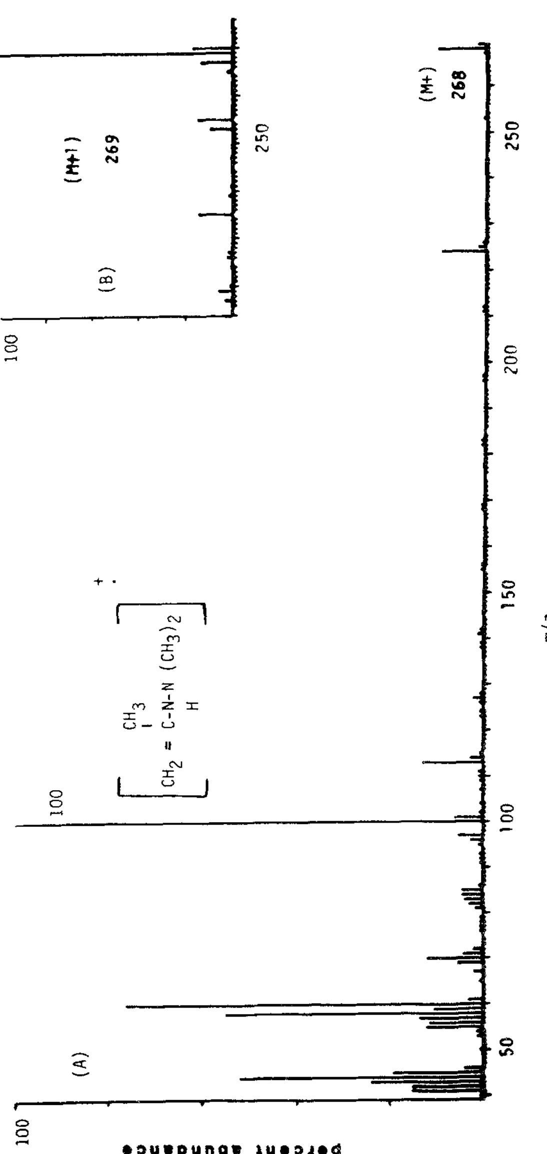


FIG. 2. Mass spectra of the *N*,*N*-dimethylhydrazone of 2-pentadecanone: (A) EI; (B) CI.

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# CUCURBITACINS
## Plant-Derived Defense Compounds for Diabroticites (Coleoptera: Chrysomelidae)

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**Abstract**—Four species of diabroticites with different host specificities are shown for the first time to sequester cucurbitacins. While all beetles fed on an artificial diet (no cucurbitacins) were readily consumed by Chinese praying mantids, a significant proportion of adult *Diabrotica balteata* (72%), *D. undecimpunctata howardi* (46%), and *D. virgifera virgifera* (24%) fed on squash fruit containing cucurbitacins B and D were rejected. Moreover, even when adults did not feed on cucurbitacins, 21–24% of *Acalymma vittatum* were rejected by the mantids which is consistent with larval sequestration of cucurbitacins. The mantids failed to learn to avoid any of the beetle species despite adverse effects associated with ingestion, i.e., uncoordination, regurgitation, etc. A cucurbitacin D metabolite accumulated and was sequestered for extended periods of time in the hemolymph of all four species. In addition, female beetles that had ingested cucurbitacins laid eggs containing substantial amounts of cucurbitacins.

**Key Words**—cucurbitacins, defense compounds, *Diabrotica* spp., *Acalymma vittatum*, insect/plant interaction, *Tenodera aridifolia sinensis*, Coleoptera, Chrysomelidae, Orthoptera, Mantidae.

### INTRODUCTION

Cucurbitacins, a group of oxygenated tetracyclic triterpenes, are efficacious allomones for the plant family Cucurbitaceae. These extraordinarily bitter compounds render the plants unpalatable and/or toxic to a variety of invertebrate and vertebrate herbivores, including humans (DaCosta and Jones, 1971; David and Vallance, 1955; Watt and Breyer-Brandwijk, 1962). At the same time, cucurbitacins (cucs) are potent kairomones for a large group of phytophagous beetles



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in the family Chrysomelidae, tribe Luperini, Old World *Aulacophora*, and New World diabroticites (Chambliss and Jones, 1966). As kairomones, cucurbitacins stimulate arrest and compulsive feeding in all species examined to date and are major factors in host-plant selection by the diabroticites (Sharma and Hall, 1973; Howe et al., 1976; Metcalf et al., 1982; Ferguson et al., 1983). Host records evidence a strong affinity of the diabroticites for the Cucurbitaceae but some *Diabrotica* species (group virgifera) are obligately associated with members of the Gramineae (Metcalf, 1979).

The universal and powerful feeding response by the diabroticites to cucs may be a vestige of their ancestral association with the Cucurbitaceae and/or reflect beneficial aspects of the ingestion of cucurbitacins (i.e., nutrition, source of precursors, predator protection, etc.). Due to their chemical characteristics, cucs are an improbable source for sterol precursors or for nutrition. Ingestion of cucurbitacins by the diabroticites, however, was first suggested by Howe et al. (1976) as possibly related to "protection against birds and other insect-feeding vertebrates."

The following study was initiated to determine if the diabroticites are capable of sequestering cucurbitacins effectively against predators and if, in addition, a predator could "learn" to avoid diabroticites that sequester cucurbitacins. Four species of diabroticites with different host specificities were examined: *Diabrotica balteata* LeConte, the banded cucumber beetle (BCB); *D. undecimpunctata howardi* Barber, the spotted cucumber beetle or southern corn rootworm (SCR); *D. virgifera virgifera* LeConte, the western corn rootworm (WCR); and *Acalymma vittatum* Fabr., the striped cucumber beetle (SCB). Adults of all four species are polyphagous, feeding primarily on pollen, while the larvae develop underground upon roots. In a practical sense, WCR larvae are restricted to corn in the field but can survive on other grass hosts (Branson and Ortman, 1970; Branson, 1971). SCB larvae have an obligate relationship with cucurbit hosts (Houser and Balduf, 1925), but the larvae of BCB and SCR are polyphagous.

In general, the major predators of the diabroticites are poorly described. Risch (1981), however, mentions the reduviid *Castolus tricolor* Champ. and gelastocorid bugs as predators of adult diabroticites in Costa Rica but adds that their effect on beetle mortality appears to be slight. Mantids have been observed in the field feeding on *D. cristata* (Ferguson, personal observation). Chinese praying mantids, shown by Berenbaum and Miliczky (1984) to be capable of learned aversion, were selected as predator for the bioassay in this study.

## METHODS AND MATERIALS

*Mantid Bioassay.* All beetles were obtained from laboratory cultures with a larval host of corn (*Zea mays* L.) for the *Diabrotica* spp. and *Cucurbita maxima* Duchesne cv. 'Blue Hubbard' for *A. vittatum*. Upon emergence, adult bee-

tles were fed either an artificial pollen diet (Branson et al., 1975) or bitter *Cucurbita andreana* × *C. maxima* fruit containing cucs B and D (ca. 1–3 mg/g fresh wt) (Rhodes et al., 1980). After a minimum of one week on a given dietary regimen, beetles were individually placed in a clear cylindrical cage (13 cm diameter × 14 cm high) with a naive adult Chinese praying mantid (*Tenodera aridifolia sinensis* Saussure). For each beetle species, adults fed on the pollen diet were individually offered to the mantid prior to single adults fed on the cuc-containing fruit being offered to the mantid. If the mantid made no predatory attempts in 30 min on the cuc-fed beetle, a fly was introduced to test for satiation. The time was recorded from first capture of the beetle by the mantid until release or complete consumption. In addition, observations were recorded on atypical mantid behavior, i.e., unsteadiness, excessive grooming, and regurgitation. A minimum of seven mantids were tested with each beetle species. All mantids were reared from eggs on a diet of flies (*Musca domestica, Phormia regina, Sarcophaga bullata*) and crickets (*Acheta domesticus*).

*Topical Application of Cucurbitacin B.* Cucurbitacin B in acetone was topically applied to the elytra of SCR beetles. After solvent evaporation, the treated beetle was offered to a mantid with forceps to prevent any possible grooming by the beetle of its elytra. The amount of cuc B topically applied was quantified by UV spectrometry (Metcalf et al., 1980).

*Elytra and Hemolymph Extraction.* Extracts of 10 pairs of elytra with methanol (3×) were centrifuged, concentrated, and applied to 0.1-mm silica gel TLC plates with fluorescent indicator. The developed chromatograms were exposed to ca. 200 adult SCR beetles, which eat the areas of the plate corresponding to cucurbitacins (Metcalf et al., 1982). Hemolymph was removed by neck puncture and applied directly to the TLC plates prior to development and beetle feeding.

## RESULTS

None of the *Diabrotica* species fed on the pollen diet were ever rejected by the mantids, nor did their consumption ever elicit any atypical behavior. In contrast, the mantids rejected 72%, 46%, and 24% of the *D. balteata, D. undecimpunctata howardi*, and *D. virgifera virgifera* fed bitter fruit (Table 1).

Rejection typically consisted of the mantid immediately flinging the beetle away (< 10 sec) after one bite of an elytron, followed by a period of marked unsteadiness, excessive grooming, and/or regurgitation. Occasionally, after long intervals of moving about on its perch and holding the beetle away from its body, the mantid would taste the beetle one or two more times prior to discarding it. In some instances, the mantid fell from its perch. Adjusted $\chi^2$ interaction values reveal significant differences in rejection rate for BCB, SCR ($P < 0.001$), and WCR ($P < 0.05$) fed bitter fruit vs. pollen but no significant differences for SCB (Table 1). There were no differences in rejection rate for male vs. female beetles of any species.

TABLE 1. CONSUMPTION/REJECTION BY CHINESE MANTIDS OF ADULT DIABROTICINA WITH AND WITHOUT EXPOSURE TO BITTER FRUIT

| Species and diet[a] | Number of Beetles: Consumed | Number of Beetles: Rejected[b] | | Beetles rejected (%) |
|---|---|---|---|---|
| *Diabrotica balteata* | | | | |
| Pollen | 33 | 0 | **[c] | 0 |
| Bitter fruit | 11 | 28 | | 72 |
| *D. undecimpunctata howardi* | | | | |
| Pollen | 40 | 0 | ** | 0 |
| Bitter fruit | 27 | 23 | | 46 |
| *D. virgifera virgifera* | | | | |
| Pollen | 37 | 0 | * | 0 |
| Bitter fruit | 41 | 13 | | 24 |
| *Acalymma vittatum* | | | | |
| Pollen | 19 | 6 | NS | 24 |
| Bitter fruit | 37 | 10 | | 21 |

[a] The pollen diet contained no cucurbitacins while the bitter fruit diet contained substantial amounts of cucurbitacins B and D.
[b] Rejection was equated with the mantid discarding the beetle intact or partially eaten.
[c] Adjusted $\chi^2$ interaction test; $**P < 0.005$; $*P < 0.05$; NS, nonsignificant at $P = 0.05$

Regardless of their adult diet, 21–24% of the SCB were rejected by the mantids. This is consistent with the larvae sequestering bitter substances from its larval host cv. 'Blue Hubbard.' Roots of 'Blue Hubbard' seedlings are extraordinarily bitter tasting and beetle bioassay confirmed the presence of large amounts of cucurbitacins.

Mantids used significantly more time to consume *D. balteata* fed the bitter fruit diet vs. the pollen diet, suggestive of the distastefulness of their prey (Table

TABLE 2. AVERAGE TIME FOR CONSUMPTION BY CHINESE MANTIDS OF ADULT DIABROTICINA WITH AND WITHOUT EXPOSURE TO BITTER FRUIT.

| Species | Average time for consumption ($\bar{X} \pm SD$ in sec)[a]: Pollen diet | Bitter fruit diet | |
|---|---|---|---|
| *Diabrotica balteata* | 126.7 ± 51.6 | 172.7 ± 103.8 | ** |
| *D. undecimpunctata howardi* | 131.7 ± 31.3 | 150.0 ± 36.6 | NS |
| *D. virgifera virgifera* | 94.3 ± 16.2 | 108.5 ± 45.8 | NS |
| *Acalymma vittatum* | 96.5 ± 26.8 | 100.7 ± 42.6 | NS |

[a] *t* test; $** P < 0.001$; NS = nonsignificant at $P = 0.05$.

2). The differences are not significant for *D. undecimpunctata* and *D. virgifera*, however. As expected, no differences in mean consumption times are evident for *A. vittatum* between the two diets due to larval exposure to cucurbitacins (Table 2).

There is no evidence to suggest that the mantids learned to recognize and avoid any of the cuc-fed beetle species. Approximately 70% of the rejected BCB and SCR fed on bitter fruit survived the mantid encounter with their major injury no more than a nick in the elytron or a missing leg segment. A lower percentage of the rejected WCR and SCB lived, most likely a function of their smaller size (SCB < WCR < BCB < SCR).

Individual variation between mantids in willingness to attack beetles was often striking. Two adult mantids never attacked any of the beetles offered to them (five trials each), while another attacked 22 SCB in a row, rejecting two, consuming 20, but regurgitating after the consumption of six beetles.

When approximately 14 $\mu$g of cucurbitacin B in acetone was topically applied to SCR beetles, the mantids consistently rejected the beetles. This strongly implicates cucurbitacins or cucurbitacin metabolites in the beetles as responsible for rejection of the beetles fed bitter fruit.

Since the mantids frequently took one bite of an elytron when they rejected the bitter fruit-fed beetles, the elytra of cuc-fed SCR beetles were clipped off and found to taste extremely bitter, a hallmark for the presence of cucurbitacins. The hemolymph, which partially circulates in the elytra, was also decidedly bitter tasting. Extraction of the elytra and hemolymph and bioassay for cucurbitacins revealed large amounts of apparently the same cucurbitacin D conjugate. After only one week of feeding on *C. andreana* × *C. maxima* bitter fruit, *D. balteata* averaged 22 $\mu$g cucurbitacin/$\mu$l hemolymph (Ferguson, unpublished data). In contrast, the elytra and hemolymph of pollen-fed SCR beetles were not bitter tasting and contained no cucs when bioassayed.

When bioassayed by TLC and beetle feeding, the hemolymph of SCR beetles with no exposure to cucs for six weeks still had quantities of cucs comparable to those fed continuously on bitter fruit. In addition, after an initial three-week feeding period on bitter fruit, all SCR beetles ($N$ = 10) on the pollen diet for nearly three months were rejected by the mantids. Thus, the hemolymph constituents are long-term storage products and not transient metabolites. Cucurbitacins, probably hemolymph-derived, were also found in the eggs of BCB, SCR, and SCB beetles fed bitter fruit as adults, as well as SCB beetles which had only larval exposure to cucurbitacins (Ferguson, unpublished data).

SCR, SCB, and WCR collected from a field having some cucurbitacin-containing *Cucurbita* plants were rejected at low rates by the mantids (20%, 20%, and 10% respectively; $N$ = 30). Bioassay of the hemolymph of field-collected beetles, however, showed that 100%, 80% and 50% of the SCB, SCR, and WCR ($N$ = 10) contained cucs or cuc metabolites. Bioassay of the elytra of field-collected beetles revealed cucs only in the SCR elytra.

## DISCUSSION

Clearly, a quantitative relationship exists between cuc sequestration and rejection by the mantid predator. The threshold below which mantids will consume the cuc-containing beetles undoubtedly varies with the given predator and the extent of its satiation. Qualitative as well as quantitative differences in cucs may also affect the efficacy of ingested cucs in protecting beetles from predators. Any of these variables could account for the contrasting results of Gould and Massey (1984), who reported that SCR fed on *Cucumis sativus* L. seedlings were consumed by three vertebrate predators (mouse, toad, quail) irrespective of the cucurbitacin content of the seedlings. Cucumber seedlings have much lower amounts of a different cucurbitacin (C) than the *Cucurbita* fruit used in this study (Ferguson et al., 1983).

The presence of cucs in the hemolymph renders any site within the beetle in contact with the hemolymph a potential site for cuc storage. Preliminary evidence has shown that, after adult feeding on bitter fruit, relatively large amounts of cucurbitacins are deposited in the eggs, which could deter egg predators, i.e., ants such as *Solenopsis geminata* F. and *Pheidole* spp. (Risch, 1981). Larvae reared on squash roots containing cucs are distinctly bitter tasting. Coupled with the characteristic reflex bleeding defenses of the larvae (Wallace and Blum, 1971), cucurbitacins may render diabroticite eggs and larvae relatively resistant to predation in the soil. In addition to deterring predators, these bitter compounds could potentially deter internal parasites. Thus, presence of the strong feeding response in all species of diabroticites examined to date reflects a tangible ecological benefit associated with the ingestion of these bitter compounds and not merely an ancestral association with the Cucurbitaceae.

Chinese mantids, shown to be capable of learned aversion by Berenbaum and Miliczky (1984), failed to learn to avoid beetles containing the toxic cucurbitacins, apparently due to the lack of recognizable aposematic coloration in the Diabroticina (Brower, 1958). Indeed, field observations reveal an absence of consistency in color patterns, especially in *Diabrotica* spp. The apparent lack of aposematic coloration in no way abrogates the effectiveness of cucurbitacins for the diabroticites, however, as approximately 70% of the rejected BCB and SCR beetles fed on bitter fruit survived the mantid encounter essentially intact. One cannot exclude the possibility that other predators can learn to avoid the cuc-fed diabroticites.

Evolution of the ability to sequester cucs may result from direct selection of the adult beetles or progeny selection of the eggs and larvae by predators. In light of the low proportion of field-collected adults rejected by the mantids, the primary advantage of ingestion of cucs may be protection of the eggs and larvae from predation. The proportion of field-collected adults rejected could substantially increase, however, in areas such as Central and South America where wild

cucurbits provide more ready access to high levels of cucurbitacins than in central Illinois. Regardless of the efficacy of cucurbitacins as a defense compound for the diabroticites, sequestration would undoubtedly be an effective adjunct to other modes of handling the toxicity of the cucurbitacins (i.e., excretion, metabolic detoxification, etc.).

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# CHEMICAL REGULATION OF POLYETHISM DURING FORAGING IN THE NEOTROPICAL TERMITE *Nasutitermes costalis*

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**Abstract**—The soldiers of *Nasutitermes costalis* communicate information about the presence and location of food by laying chemical trails of sternal gland secretion. These trails first recruit additional soldiers, and as the number of soldiers contacting food and returning to the nest increases, trail pheromone concentration increases, and workers are recruited. This polyethic pattern of recruitment does not appear to depend on qualitative (caste-specific) properties of soldier and worker sternal gland secretions, but rather on quantitative differences in pheromone production between castes. Large third-instar workers have significantly greater sternal gland volumes than soldiers, and glands of approximately equivalent size have approximately equivalent recruitment effects. The recruitment and orientation effects of artificial trails prepared from worker sternal glands can be mimicked by increasing the concentration of soldier sternal gland pheromone.

**Key Words**—*Nasutitermes costalis*, Isoptera, Termitidae, termite, trail pheromone, foraging, social behavior, chemical ecology, communication.

## INTRODUCTION

Foraging in termites is a social procedure: the activities of hundreds or thousands of individuals are coördinated by trail pheromones that stimulate foragers to leave the nest and orient them to a food find. Termite trail pheromones originate in the sternal gland, which is composed of a group of modified epidermal cells on the fifth abdominal sternite (Quennedey and Leuthold, 1978; Stuart and Satir, 1968). After a termite locates food, it returns to the nest in trail-laying posture, discharging sternal gland pheromone. Other termites are subsequently recruited. Although soldiers and workers may possess well-developed sternal



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glands, soldiers are specialized for defense (they do not feed) and typically play a protective role during foraging. This generalized description of termite foraging appears to be true for most species of termitids that have been studied and primarily involves communication between workers.

However, there have been reports suggesting that soldiers of some species have a more active part in foraging. Beaumont (1889) and Andrews (1911) noted that soldiers of species of *Nasutitermes* preceded workers in many social activities. The descriptions and experimental studies of foraging behavior in *Nasutitermes corniger* of Stuart (1969, 1981) support these observations, and suggest that nasute soldiers are involved in foraging. Traniello (1981) subsequently showed that soldiers of *N. costalis* function as scouts, preceding workers in foraging. In this paper we describe in detail recruitment communication in the neotropical termite *Nasutitermes costalis*, focusing on the role of the soldier caste in the organization of foraging and the nature of the signals involved in intercaste communication.

## METHODS AND MATERIALS

*Laboratory Arrangements. Nasutitermes costalis* is one of the most widespread nasutitermitine species in the New World tropics, ranging in distribution from Cuba to Bolivia and northern Brazil. This species forms arboreal carton nests composed of fecal material and debris. The majority of the data of the present study were obtained from experiments and observations on a single colony of *N. costalis* collected in 1973 on the island of Dominica in the Lesser Antilles. The colony has adapted well to the laboratory setting and has continually expanded its nest, which lies in a 40 × 30 × 15-cm pan within a 78-liter plastic garbage can placed on top of a foraging surface made of glass plates. The top of the can is covered with a 25 × 25-cm sheet of glass. Holes in the bottom of the container permit access to the foraging area, made up of 10 interconnecting 46 × 61-cm glass plates (total surface area = 2.8 $m^2$) supported on mineral oil traps, comprising a rectangular surface 230 × 122 cm. Partially decayed birch was used as food and, to provide moisture, large glass test tubes (200 mm in length, 38 mm inner diameter) filled with water and fitted with a tight cotton plug were placed on the foraging plates.

*Preparation of Glandular Extracts.* To remove the sternal gland, the abdomen of a termite was gently pulled free of the thorax and eviscerated. Soldiers were first immobilized by chilling to prevent discharge of cephalic gland secretion. Sternites and tergites were then separated, and the fifth sternite, which bears the sternal gland, was teased free of adjacent segments and crushed in 10$\mu$l 100% ethanol in a Kontes extract vial. Usually 10 gland preparations were pooled to decrease individual variation. Artificial trails of various extract composition and concentration were drawn on chromatography paper.

All sternal gland extracts were standardized at a concentration of one gland/ 10 $\mu$l solvent. Extracts were kept on ice and used immediately following preparation.

*Histology.* Termites were fixed in alcoholic Bouin, embedded in Paraplast, and 5-$\mu$m sections were cut with a rotary microtome. Specimens were stained with Azocarmine.

*Sternal Gland Volume Determination.* Sternal gland volumes were estimated from freshly dissected glands. Gland length and thickness were measured with a reticle eyepiece at 50× on a Wild stereomicroscope. The sternal gland is somewhat ellipsoidal in shape, and its volume was approximated using the formula for the volume of an ellipse [$V = 4/3$ (length) (width) (thickness)].

The designation of castes (large worker, LW; small worker, SW) and instar number is in accordance with McMahan (1970).

*Sternal Gland Pheromone Bioassays for Recruitment.* Bioassays for recruitment were performed using groups of termites (subnests isolated from the main colony) of fixed and approximately equal numbers of workers and soldiers. Only subnests of more than 50 termites (total) were used. Termites were placed on filter paper under Petri dish covers (100 mm diameter) and were acclimated for at least 1 h after transfer from the main colony. One side of the Petri dish cover was gently raised and propped up with cotton to prevent disturbance from air currents. Ten microliters of extract solution were used to draw a 10- or 25-cm trail with a Hamilton syringe (701-N, 10 $\mu$l), tracing a sinusoidal line made with pencil while evenly dispensing the solution. All recruitment trails were drawn on filter paper and carefully placed next to a subnest to allow termites access to the trails. The beginning of the trail was carefully positioned within 1 cm of the termites in the subnest. Responses were recorded during a 3-min time interval. This time period was chosen because the rate of recruitment to sternal gland extract trails rapidly decreases beyond three minutes. A positive response was defined as a following response of 5 cm or greater. Each subnest was used only once per day, and no subnest was used for longer than three days. At all times we attempted to minimize mechanical disturbance.

*Bioassay for Orientation.* Orientation effects were tested in a Y-shaped configuration by drawing a 10-cm trail of test extract or an aged sternal gland trail at a 45° angle from the terminus of a 10-cm long, fresh sternal gland extract trail. The beginning of the fresh sternal gland extract trail was placed within 1 cm of termites in a subnest. This trail was the stem of the Y. A solvent control trail was drawn at a 45° angle from the test trail creating a choice of trails for termites reaching this junction. The solvent and test trails, therefore, were the branches of the Y. We recorded only the response of termites that contacted the trail chemotropotactically by sweeping the antennae and swinging the body through an arc of about 60° at the junction of the two trails. An orientation response was recorded when following occurred for at least 5 cm. Termites were aspirated from the test trail after responses were recorded. We did not observe termites making any contribution to the trail by active trail laying.

## RESULTS

### *Description of Foraging Behavior and Patterns of Recruitment to Food*

Exploration of a new foraging area for food is conducted by groups of nasutes that venture from the nest or termini of existing covered trails. Leadership in these groups is inconsistent; soldiers at the front most often turn back after traveling a few centimeters and are replaced by others. Soldiers frequently are observed pressing their abdomens to the substrate, in trail-laying posture. This behavior is observed in soldiers leaving and returning to the nest, and soldiers appear more cautious and move at a slower pace as they move away from the nest. At first the groups of soldiers have little directionality, and the movement pattern resembles the motion of the pseudopods of an ameba. Exploration continues in this fashion until food is encountered. Soldiers contacting the food return to the nest in trail-laying posture, and the initial response is the recruitment of additional soldiers. Soon, the exploratory "pseudopods" coalesce into a single trail. Some soldiers continue exploring adjacent areas, while others adopt guard positions at the food or on the flanks of the trail. Few workers are recruited during this phase of foraging (Table 1).

The second phase of foraging is marked by the recruitment of workers, which increases rapidly after the first few workers return from the food. Chiefly LW2, LW3, and SW2 workers forage, as McMahan (1970) and Jones (1980) have described. During this phase, soldier and worker recruitment increases rapidly (Table 1), and the soldier–worker ratio at the food decreases sharply. The foraging trail now begins to take on the form characteristic of many nasute species, as soldiers align themselves in defensive positions along its periphery, and worker traffic appears channeled within the soldiers' flanks.

During the final phase of foraging organization, the width of the trail in-

TABLE 1. RECRUITMENT RATES (MEAN NUMBER OF TERMITES/MINUTE ± STANDARD ERROR) DURING FIRST THREE PHASES OF FORAGING[a]

| Phase | Caste | |
|---|---|---|
| | Soldiers | Workers |
| 1. Soldier recruitment and early worker recruitment (< 1 hr) | 34.4 ± 1.8 | 2.4 ± 0.5 |
| 2. Worker recruitment (~3 hr) | 19.8 ± 1.1 | 37.0 ± 1.4 |
| 3. Trail establishment | | |
| Early (~15 hr) | 11.6 ± 1.2 | 69.4 ± 2.3 |
| Late (~30 hr) | 8.2 ± 0.9 | 136.2 ± 8.4 |

[a] Data based on 10 consecutive, 1-min samples taken during each phase. Food source was placed 50 cm from an existing foraging area.

creases to accommodate the bidirectional traffic of workers; few soldiers are recruited while worker recruitment continues to increase (Table 1). Fecal pellets (pavé) accumulate on the trail. The trail is then gradually enclosed in an arcade or shelter tube, semicircular in cross-section, composed of feces.

The differential recruitment of soldiers and workers suggests that soldiers function as scouts and communicate the location of food to workers. Although this recruitment pattern has been suggested in other nasute species, it has not been determined if differential caste recruitment represents soldier-organized foraging or is simply a temporal characteristic of nasute foraging behavior. To distinguish between these two possibilities, we separated soldier scouting behavior from soldier recruitment of workers by controlling food availability during the exploratory phase of foraging. A clean 46 × 61-cm glass plate was placed adjacent to one of the occupied foraging plates of the main colony, on which soldiers had begun exploratory behavior. The two glass plates were in full contact along their 46 cm width. After contacting the new area, the number of soldiers soon increased, indicating that additional soldiers were recruited by scouting soldiers. However, workers were not recruited in appreciable numbers until a piece of birch was placed on the plate (Figure 1). This suggests that exploration and food recruitment are distinct phenomena and that the presence of food, but not new foraging space, is communicated by soldiers to workers. Furthermore, to unequivocally establish that intercaste communication is occurring, workers were prevented (by aspiration) from returning to the nest for 30 min after food was present. Worker recruitment did not decrease, suggesting that a communicative exchange continued between soldiers and workers (Figure 1).

### *Chemical Signals Mediating Intercaste Communication*

During food recruitment, both soldiers and workers were observed pressing their abdominal sternites to the substrate while traveling between the nest and the food source. Histological sections showed that soldiers, as well as small and large worker castes, possess a well-developed sternal gland. Because of the differential recruitment pattern of soldiers and workers during the early stages of foraging, we tested the possibility that caste-specific components of the trail pheromone were responsible for differential caste recruitment. Artificial trails, 25 cm in length, composed of one sternal gland (soldier or worker) in 10 $\mu$l ethanol were placed near groups of termites in a binary choice situation. Trails prepared from glands of either caste diverged at a 90° angle. If the sternal gland pheromones are caste-specific, then soldiers and workers should show a preference for the trail of their caste. However, more soldiers and workers were recruited by a worker trail than a soldier trail, and more individuals of both castes followed the trail for its entire length.

In ten replicates, a total of 74 soldiers and nine workers were recruited by a 25-cm artificial trail prepared from a soldier sternal gland extract, whereas

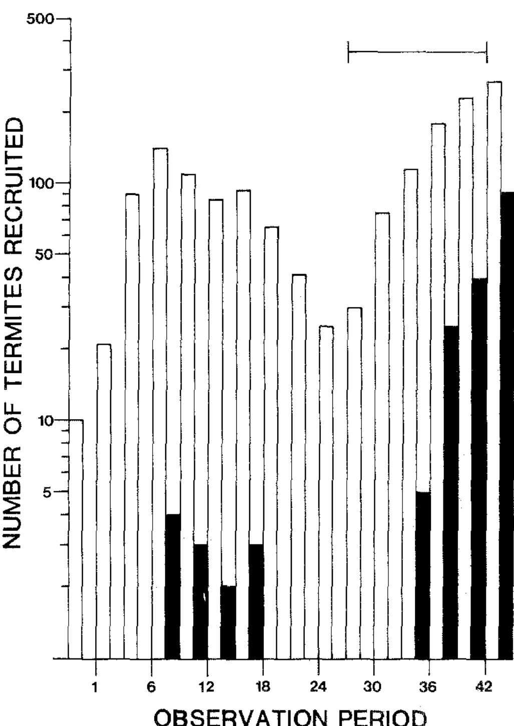


FIG. 1. Pattern of recruitment of soldiers and workers to a new food source. Counts were taken during consecutive 2-min observation periods. Data from each third period are illustrated. Horizontal black bar indicates the time during which workers were prevented from returning to the nest. At the beginning of this period food was placed in the foraging arena. □ = soldiers. ■ = workers.

252 soldiers and 205 workers were recruited by a similar worker sternal gland trail. Also, the following response of termites on each type of trail was different. Forty-two percent of soldiers and 56% of workers followed a soldier trail for a distance of less than 10 cm, and 16% of soldiers and 11% of workers followed it for 20–25 cm. In response to a worker sternal gland trail only 14% of soldiers and 16% of workers turned back at a distance of 10 cm or less, whereas 60% of soldiers and 66% of workers followed for 20–25 cm. These results indicate worker-generated trails have a greater recruitment potency than soldier-generated trails.

*Qualitative and Quantitative Differences in Soldier and Worker Trail Pheromones*

*Recruitment Effects.* The effectiveness of the extracts of glands of each caste as recruitment pheromones was assayed by presenting termites with arti-

ficial trails of various composition. The persistence of the recruitment effect and soldier and worker responses were also tested. Traniello (1982a) showed that soldier sternal gland secretions are less effective in recruiting both soldiers and workers than are worker secretions. Furthermore, the recruitment effects of extracts of soldier glands decay more rapidly than extracts of worker glands. Trails aged 15 min under dim red light at 26°C had little effect in eliciting a recruitment response from either caste (Traniello, 1982a,b). The lack of caste specificity of sternal gland secretions and qualitative similarity of the activity of the trail pheromones suggest that the behavioral differences between castes during foraging organization may be a concentration-dependent phenomenon. Soldiers and workers may differ in the amount of secretion produced, which might be reflected in gland volume. We dissected and measured the volume of glands of soldiers, small second-instar workers, and large third-instar workers (castes most frequently seen on foraging trails). Although all three castes show overlap in gland volume (Figure 2), the LW3 gland volume (906 ± 255 $\mu m^3$, mean ± standard deviation) is significantly greater than soldier gland volume (409 ± 120 $\mu m^3$; $t = 9.38$, $P < 0.001$) and SW2 gland volume (377 ± 194 $\mu m^3$, $t = 19.5$, $P < 0.001$). Soldier and SW2 gland volumes are not significantly different ($t = 0.21$, $0.1 < P < 0.2$).

We next used this information to test if soldier and worker sternal gland secretions are quantitatively or qualitatively different. We tested for differences

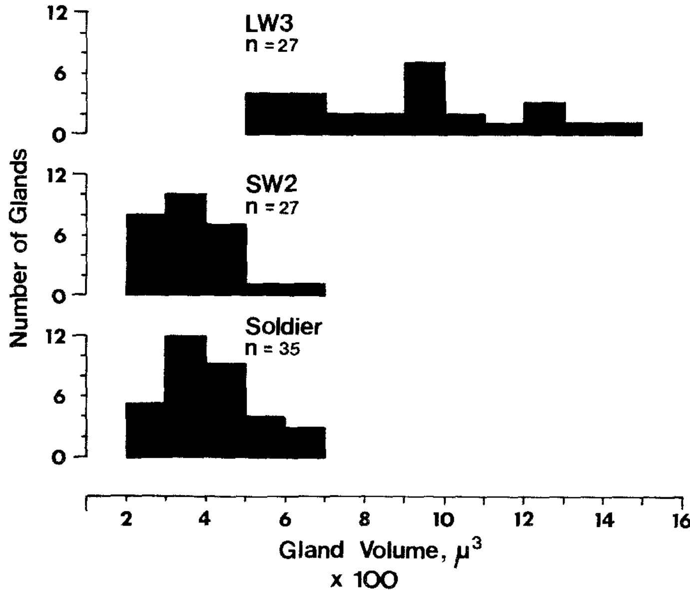


Fig. 2. Frequency distribution of gland volumes of soldiers, small second-instar workers, and large third-instar workers. $N$ = sample size.

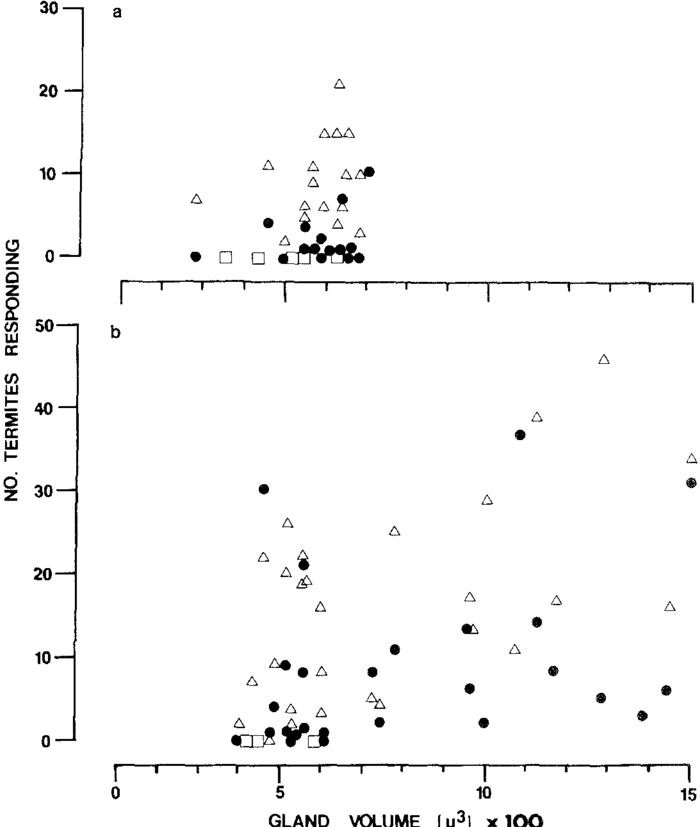


FIG. 3. Relationship between gland volume and caste response. (A) Recruitment response to extracts of soldier sternal glands. (B) Recruitment response to extracts of large third-instar worker sternal glands. △ = number of soldiers responding; ● = number of workers responding; □ = no response, either caste.

in the recruitment ability of LW3 and soldier sternal glands. If there are qualitative differences between soldier and worker sternal gland pheromones, then castes should differ in response to artificial trails made from glands of equal volume from different castes. For the range of soldier sternal glands extracted, there was no significant correlation between gland volume and response and significantly more soldiers than workers responded to soldier sternal gland trails (Mann-Whitney U test, $P < 0.001$; Figure 3A). For trails prepared from worker glands, there is a significant correlation between gland volume and recruitment response of soldiers ($r = 0.38$, $P < 0.05$) and workers ($r = 0.57$, $P < 0.01$; Figure 3B).

We considered 600 $\mu m^3$ as the upper limit of soldier/LW3 overlap in gland

TABLE 2. RECRUITMENT EFFECTS OF SOLDIER AND LARGE THIRD-INSTAR WORKER STERNAL GLANDS OF APPROXIMATELY EQUAL VOLUME[a]

| Caste | $N$ | Gland volume ($\mu m^3$) (mean ± standard error) | Response (mean ± standard error) | |
|---|---|---|---|---|
| | | | Soldiers | Workers |
| Soldier | 20 | 580 ± 20.0 | 7.4 ± 1.5 | 1.5 ± 0.6 |
| Worker | 20 | 520 ± 20.0 | 8.7 ± 1.9 | 4.5 ± 1.8 |

[a] Although more workers on average recruit to a worker trail than a soldier trail, this difference is not statistically significant. Twenty glands of each caste were used, one gland for each replicate per caste, for a total of 20 replicates.

volume. For gland volumes less than or equal to 680 $\mu m^3$, significantly more soldiers than workers are recruited. This pattern also holds for gland volumes greater than 680 $\mu m^3$. Furthermore, when we examined worker and soldier responses to glands of approximately equal volume dissected from each caste, we found no significant difference in the mean number of soldiers ($U_{20,20} = 209$; NS) or workers ($U_{20,20} = 226.5$; NS) recruited (Table 2). However, significantly more soldiers than workers were recruited on artificial trails made from extracted soldier ($P < 0.025$) and worker ($P < 0.001$) glands. These results suggest that soldiers have a lower threshold sensitivity for trail pheromone detection and that the differential caste recruitment observed during foraging organization is a concentration-dependent (quantitative) phenomenon.

*Orientation effects.* The sternal gland secretion of *N. costalis* contains components that regulate recruitment and orientation responses (Traniello, 1982a). Although the recruitment effect of a trail is ephemeral, its orienting properties are persistent. We examined caste differences in the orientation properties of sternal gland pheromone. (The assays for orientation are described in Materials and Methods). Trails made from soldiers' and workers' sternal glands overlapping in volume were aged 15 min. Table 3 shows that there was no significant difference in the number of soldiers or workers orienting to trails made from glands of either caste. However, significantly more soldiers followed orientation trails prepared from glands of their own caste ($t = 6.6$, $P < 0.001$, arc sin transformation test for equality of two percentages), as well as trails prepared from worker glands ($t = 3.9$, $P < 0.001$). This suggests that soldiers also have lower thresholds for detecting the orientation component of the trail pheromone.

### *Concentration Dependence of Recruitment and Orientation Effects of Soldier Sternal Gland Pheromone*

The hypothesis that soldier and worker sternal gland secretions are quantitatively different predicts that increasing the concentration of pheromone pro-

TABLE 3. ORIENTATION RESPONSES OF SOLDIERS AND WORKERS TO ARTIFICIAL TRAILS MADE FROM STERNAL GLANDS OF SOLDIERS AND LARGE THIRD-INSTAR WORKERS[a]

| | Orientation response | | | |
|---|---|---|---|---|
| | Soldiers | | Workers | |
| Gland tested | $N$ | % following | $N$ | % following |
| Soldier | 38 | 60.5 | 26 | 53.8 |
| Worker | 48 | 35.4 | 30 | 26.7 |

[a]Gland size was kept approximately equal, ranging in volume from 547–625 $\mu m^3$ for soldiers and from 554 to 639 $\mu m^3$ for workers. Trails were aged for 15 min and tested in competition with a solvent trail. Three replicates were performed for each gland, and results were pooled. $N$ = number of termites tested. The orientation response is expressed as the percent of individuals tested that follow the aged trail.

duced in the caste with the smaller gland volume (soldiers) should increase the response of the caste having the higher response threshold (workers). To test this prediction, we varied the number of soldier sternal glands extracted in 10 $\mu$l ethanol used to make a 25-cm-long artificial trail and recorded the recruitment and orientation responses of each caste. Table 4 shows that the recruitment and orientation responses of each caste varied in response to increasing soldier sternal gland pheromone concentration. Due to the large variance in soldier response, there is no significant difference in soldier recruitment as pheromone concentration increases from two to six glands per trail. However, for the same concentration increase, the number of workers recruited is significantly higher ($P < 0.001$, Mann-Whitney U test). Caste responses are significantly different at two glands/trail ($P < 0.001$) and four glands/trail ($P < 0.001$) as more soldiers than workers respond, but there is no significant difference in response at six glands/trail.

TABLE 4. RECRUITMENT AND ORIENTATION RESPONSES OF SOLDIERS AND WORKERS TO VARYING CONCENTRATIONS OF SOLDIER STERNAL GLAND PHEROMONE[a]

| | Recruitment | | Orientation | |
|---|---|---|---|---|
| $N$ | Soldiers | Workers | Soldiers | Workers |
| 2 | 11.6 ± 5.1 | 0.6 ± 0.4 | 19.9 ± 9.3 | 13.2 ± 5.7 |
| 4 | 18.0 ± 1.7 | 4.4 ± 1.4 | 47.6 ± 6.9 | 53.7 ± 11.0 |
| 6 | 30.0 ± 6.3 | 22.4 ± 10.4 | 65.6 ± 5.4 | 70.2 ± 5.2 |

[a]The mean and standard error of the number of termites recruited by a fresh trail and following a 15-minute-aged trail (see Methods and Materials) are given. $N$ = number of soldier sternal glands. Ten replicates for each concentration for recruitment and orientation tests.

Orientation responses of both soldiers and workers significantly increase as pheromone concentration varies from two to six glands ($P < 0.02$, $P < 0.001$, respectively, for each caste). There is no significant difference between soldier and worker orientation responses at any concentration, although at two glands/trail more soldiers respond, whereas at four and six glands/trail worker response is somewhat higher. It seems likely that caste differences in response, which we were able to demonstrate at lower concentrations, are masked due to the relatively high pheromone concentrations in our experimental situation. These studies indicate that it was possible to chemically "mimic" the recruitment and orientation effects of a worker sternal gland extract by increasing the concentration of soldier sternal gland pheromone.

## DISCUSSION

In the Nasutitermitinae, sternal gland secretions are involved in food recruitment and defensive behavior (reviewed in Prestwich, 1983). Differences in caste response to sternal gland pheromone have been reported in *Nasutitermes corniger* (Stuart, 1981, and references therein) and *Trinervitermes trinervoides* (Tschinkel and Close, 1973), and appear to be involved in systems of foraging organization similar to what we have described in *N. costalis* (Traniello, 1981, 1982a,b). Our experimental studies suggest that the pattern of recruitment of soldiers and workers is not regulated by qualitative differences in the sternal gland pheromones of each caste. In behavioral assays of the recruitment effects of glands of equal volume from different castes, worker sternal glands, on average, recruited more workers than did soldier sternal glands of equivalent volume. However, this difference was not statistically significant, and the difference in mean response was due to an unusual response in only two of 20 trials.

Our arguments are based on the assumption that gland volume is related to its concentration of pheromone. Although our preliminary chemical analysis of the components of soldier and worker sternal gland pheromones show several common constituents, we are unable at this time to quantify the similarities and differences in terms of trail pheromone chemistry (Prestwich and Traniello, unpublished). However, our present studies demonstrate significant correlations between gland volume and recruitment activity (Figure 3B), suggesting that our assumption of a correlation between gland volume and pheromone concentration is valid.

Differences in caste response to sternal gland secretion appear to be based on two factors: (1) caste differences in the sensitivity thresholds of chemoreceptors involved in trail pheromone perception, and (2) differences in the amount of pheromone produced in the sternal glands of soldiers and workers. Regulation of pheromone deposition by soldiers does not appear to play a role in differential caste recruitment because whole soldier gland extracts are not

able to recruit workers. This is based on the assumption that the concentration of an artificial trail composed of a whole gland extract exceeds that of a natural soldier-generated trail. Differences in chemoreceptor thresholds are reflected in our behavioral assays of caste response to varying concentrations of soldier sternal gland pheromone, which produces a concentration-dependent response in workers but has a recruitment effect in low concentrations that is primarily restricted to soldiers. Differences in sensory physiology and pheromone production may underlie patterns of caste polyethism that are prominent in nasute termites, as Stuart (1975) has suggested. Of course, without appropriate neurophysiological tests, we do not know if the behavioral differences are due to peripheral receptor thresholds or some central integrative process.

Soldiers are the first individuals to leave a gallery or the nest in both foraging or defensive situations. Both foraging and defense are socially organized, and sternal pheromone serves as an attractant and an arrestant for soldiers (Stuart, 1981; Traniello, 1981). The fact that soldiers are more responsive to sternal gland pheromone than workers seems to be a correlate of their defensive specialization. As many observers have noted, soldiers precede workers in nearly all activities. By laying relatively weak trails that initially affect only soldiers, a high soldier–worker caste ratio, which enhances defense, is assured.

In *Trinervitermes bettonianus*, Oloo and Leuthold (1979) describe a mass foraging system in which food-finding workers lay chemical trails about five times stronger than the exploratory trails generated by scouting workers, and suggest that two types of trails exist: "basic trails" (for orientation) and "recruitment trails" for stimulating nestmates to forage. In *Nasutitermes costalis*, both excitatory and orientation information are chemically coded in the sternal gland pheromone of both castes, and soldier-generated exploratory trails appear to be quantitatively different from worker-generated trails. Although pheromone deposition may be facultatively regulated by individual soldiers, it seems more likely that it is the cumulative effect of trail reinforcement by soldier groups that initiates worker foraging.

Soldier sternal glands are smaller on average than female worker sternal glands, and increasing the concentration of soldier trail pheromone on an artificial trail can mimic the effects of a trail made from an extract of a worker sternal gland. In *N. costalis*, a soldier-generated trail has roughly half the strength of a trail generated by a large worker. Similar patterns may occur in *N. corniger* (Stuart 1981). Soldier-organized foraging has not been described in *Trinervitermes*, and the quantity and properties of the sternal gland secretions of soldiers and workers appear to differ. In *T. bettonianus*, soldier sternal glands are almost vestigial (Leuthold and Lüscher, 1974), whereas in *T. trinervoides* soldier-generated trails are about one sixth as strong as worker trails. Also there is no preference by either caste for their own trails in this species, suggesting a quantitative difference in sternal gland volumes. In *T. bettonianus* workers locate

new food sources, and there is a correlation between sternal gland development and participation in foraging (Oloo, 1981).

Coles (1980) and Mill (1982) have correlated caste ratios, nest structure, and the nature of soldier defense with foraging ecology in a diverse array of neotropical termite species. Species with easily penetrated nests which have workers that forage epigaeically invest in large numbers of soldiers that use defensive chemicals for protection. Some species of *Nasutitermes* show what appears to be soldier-initiated foraging, similar to what we have described in *N. costalis* (Mill, personal communication). Surface foraging and predator pressure are likely to have been important selective agents in the evolution of these polyethic, pheromonally coordinated behaviors in termites.

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# EFFECT OF EMPTY COMB ON DEFENSIVE BEHAVIOR OF HONEYBEES

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**Abstract**—Honeybees in colonies with 6.36-$m^2$ surface area of empty comb responded faster to moving targets and stung the targets more often than bees from colonies with 3.18-$m^2$ surface area of empty honeycomb. The two groups did not differ significantly in speed of response to alarm pheromone or in number of bees defending the colony. Volatiles from the comb are suggested as primer pheromones for defensive behavior.

**Key Words**—Honeybees, *Apis mellifera,* Hymenoptera, Apidae, colony defense, stinging, comb volatiles, pheromones.

## INTRODUCTION

Rinderer and Baxter (1978) reported that 4.06 $m^2$ of comb surface area (CSA) of empty beeswax comb suitable for the storage of honey added to a colony of honeybees, *Apis mellifera,* during a period of intensive nectar secretion and availability, significantly increased the amount of nectar gathered and stored as honey, over the quantity stored by a colony given only 1.88 $m^2$ CSA of empty comb. A possible mechanism for the effect of empty comb on honey production was demonstrated by Rinderer (1981). Caged adult worker honeybees hoarded (stored in comb, see Free and Williams, 1972; Kulincevic and Rothenbuhler, 1973) sucrose solution at an increased rate in the presence of volatiles from warm (35°C) empty comb. Further work (Rinderer, 1982) proposed that differing levels of volatiles from empty comb shifted aspects of honeybee nectar foraging according to seasonally varying conditions of nectar availability.

Results of an experiment comparing colony defense by Africanized and European bees in Venezuela (Collins et al., 1982) led us to postulate that empty comb might also influence defensive behavior. Data from this comparison of



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ecotypes, analyzed for the effect of empty comb, and from a second experiment designed specifically to determine the influence of empty comb on defensive behavior are presented here.

## METHODS AND MATERIALS

For both experiments, a standardized test (Collins and Kubasek, 1982) was used to assess the level of defensive behavior of each colony. Prior to testing, two pictures were taken: one of the bees on the colony around the entrance, and one of the bees flying in front of the colony. The actual test began when 0.8 ml of an artificial alarm pheromone [isopentyl acetate in paraffin oil 1:100 (v/v)] was sprayed above the colony entrance. The time when recruited bees began to issue from the hive was recorded. At 30 sec after spraying, a second pair of photographs was taken, and the colony was given a physical jolt by hitting it with a glass marble (1.9 g, 2.3 cm diam) propelled by a slingshot. A third pair of photographs was taken at 60 sec and two blue suede targets (5 × 5 cm) were moved into place, one 2–6 cm in front of the entrance and one 25 cm farther away. These targets were clipped to the arms of a battery-operated device which swung them up and down through 20 cm about 120 times/min during the 60-sec to 90-sec interval. The time at which the first bee was seen on a moving target was recorded. At 90 sec, the targets were removed and final photographs made. A pair of targets was used only once and replaced for each test sequence. At a later time, the number of stings adhering to each target and the number of bees in each picture were counted.

*Ecotype Comparison Experiment.* Ten colonies each of Africanized and European bees (900 g; 8500–10,000 workers) were established in either 20-liter (½ standard brood chamber) or 40-liter (standard brood chamber) hives with equal brood and honey and 0.52 $m^2$ and 1.56 $m^2$ empty CSA, respectively. After six weeks (during which an unrelated experiment was performed), the colonies were transferred to new, three-comb (43 × 20 cm), 20-liter nucleus colonies with 3-cm entrances and established in one new apiary location near Maturin, Monagas, Venezuela. At the time of transfer, the populations of adult bees and brood were judged to be still equal. On the third and fourth days, the colonies were evaluated using the standardized test, serving as a control for a larger experiment (Collins et al., 1982) comparing colony defense by the two ecotypes. Data were analyzed for ecotype, previous hive volume, and test day by a three-way analysis of variance.

*Empty Comb Experiment.* Twenty-four colonies of European bees were chosen on the basis of approximately equal numbers of bees (20,000–30,000), equal size brood nests, and equal honey and pollen stores. Each colony was derived from a queen of open-mated mixed commercial stock. These colonies were established at one apiary location near St. Gabriel, Louisiana, after the

major honey production season. The colonies were randomly assigned to a treatment group and given empty honey storage comb with 3.18 $m^2$ CSA (three shallow supers) or 6.36 $m^2$ CSA (six shallow supers) in addition to their brood nest.

After six weeks (sufficient time for an effect to be significant, as seen in the first experiment), each colony was tested on three days between 10 AM and noon using the standardized test (test 1). Then the honey storage comb was removed, any collected honey was removed, and the two CSA treatment levels were reversed on the same set of 24 colonies. After one week, the colonies were retested three times, again between 10 AM and noon (test 2). A third replicate of three tests was made six weeks after treatment reversal (test 3). Data were transformed to $\log_e$ and analyzed by least-squares analysis of variance.

## RESULTS AND DISCUSSION

*Ecotype Comparison Experiment.* Means of the seven measurements of defensive behavior for each level of empty honey storage comb surface area are presented in Table 1. There was no difference in time to react to the artificial alarm pheromone. However, when a moving target was presented, bees that had been in a hive with more CSA responded approximately twice as fast as bees from the units with less CSA. There were also twice as many stings in targets attacked by these bees.

The influence of more CSA was also seen in the number of bees responding

TABLE 1. MEASUREMENTS OF COLONY DEFENSIVE BEHAVIOR BY HONEYBEES PREVIOUSLY IN 20-LITER OR 40-LITER COLONIES (0.52 $m^2$ AND 1.56 $m^2$ CSA, RESPECTIVELY) (ECOTYPE COMPARISON EXPERIMENT)[a]

| | Previous hive volume (liter) | | |
|---|---|---|---|
| Measurement | 20 | 40 | $F^b$ |
| Time to react (s) to: | | | |
| Pheromone | 14.9 | 13.5 | 0.3 |
| Target | 8.5 | 4.4 | 4.9* |
| Total No. stings | 4.7 | 9.5 | 7.9** |
| No. of bees on colony front at: | | | |
| Pre | 3.2 | 2.5 | 0.6 |
| 30 sec | 4.3 | 9.4 | 11.0** |
| 60 sec | 5.6 | 12.2 | 6.7* |
| 90 sec | 6.3 | 7.7 | 0.9 |

[a] Values are means from 10 colonies each tested twice.
[b] $df = 1$; $*P < 0.05$; $**P < 0.01$.

after the field test began, at 30 and 60 sec, but not at 90 sec. The more defensive Africanized bees exhibited similar differences when compared with European bees in the small units (Collins et al., 1982). It was proposed that the lack of difference at 90 sec was due to an incomplete count of defending bees. Africanized bees tended to fly off the entrance and attack more readily and, therefore, many did not show up in the photograph. Europeans did not fly as readily. A similar difference might exist between high CSA bees when compared to low CSA bees.

The results of this experiment indicate that honeybee defensive behavior is affected by the presence of empty comb and that the effect remains for a period of time after the comb is removed. Similar stimulation by empty comb has also been shown to alter hoarding behavior by caged adult workers (Rinderer and Baxter, 1979) and honey production in field colonies (Rinderer and Baxter, 1978).

*Empty Comb Experiment.* The measurements of colony defensive behavior by field colonies with 3.18 $m^2$ and 6.36 $m^2$ CSA are shown in Table 2. Six weeks after empty comb was placed on the colonies (combined tests 1 and 3), bees from nests with greater CSA responded to the moving targets faster and stung them twice as much as did bees from small CSA nests. There were no significant differences between treatments for time to react or the number of bees reacting. However, the values for 6.36 $m^2$ CSA colonies were consistently indicative of greater responsiveness. At one week, it appears that the reversed CSA treatment levels are already affecting the expression of the behavior, as the two treatments are no longer significantly different for any component of the behavior.

In a comparison of the defense test results one week after treatment reversal (test 2) with the results of testing six weeks after treatments were applied (reversed, tests 1 and 3), it appears that the colonies were disrupted by the treatment reversal. More bees are present on the entrance and more stings were counted for test 2 than for tests 1 and 3. A week is more than sufficient time for a colony to settle down after being worked (physically disturbed), and no major differences in temperature, humidity, or foraging behavior were observed. Thus, it seems likely that this difference in behavior may reflect the unsettling effect of having the comb treatments reversed and altering the levels of comb volatiles present.

Rinderer (1981) concluded that it was the volatiles from empty comb at temperatures similar to that of the normal brood nest which increased hoarding behavior, rather than any actual contact with the combs. He proposed that these volatiles are pheromones incorporated into the comb by the bees themselves. Our results show that the presence of empty comb in a colony enhances the level of defensive behavior. Comb volatiles may function as primer pheromones in defensive behavior, altering the physiology of worker bees such that they are more responsive to primary stimuli eliciting colony defense (Collins et al., 1980).

TABLE 2. MEASUREMENTS (LEAST-SQUARES MEANS ± STANDARD ERROR) OF DEFENSIVE BEHAVIOR BY HONEYBEES EXPOSED TO TWO LEVELS OF EMPTY COMB[a]

| Behavioral component | Test[b] | Comb surface area ($m^2$) 3.18 | 6.36 | $F^c$ |
|---|---|---|---|---|
| Time(s) to react to: | | | | |
| Pheromone | 1 | 9.9 ± 1.0 | 8.9 ± 0.7 | |
| | 2 | 10.6 ± 0.7 | 9.4 ± 0.9 | 1.23 |
| | 3 | 9.7 ± 1.0 | 7.9 ± 0.8 | |
| | 1 + 3 | 9.7 ± 0.6 | 8.6 ± 0.5 | 1.15 |
| | | $F^d$ 2.63 | | |
| Target | 1 | 8.2 ± 1.0 | 6.4 ± 0.8 | |
| | 2 | 7.9 ± 0.9 | 7.5 ± 1.0 | 0.77 |
| | 3 | 8.8 ± 1.0 | 5.5 ± 0.8 | |
| | 1 + 3 | 8.5 ± 0.6 | 6.0 ± 0.6 | 8.85** |
| | | $F^d$ 0.16 | | |
| Total No. stings | 1 | 9.5 ± 1.5 | 18.3 ± 2.7 | |
| | 2 | 13.4 ± 2.4 | 20.4 ± 3.5 | 2.39 |
| | 3 | 4.2 ± 1.0 | 12.5 ± 2.2 | |
| | 1 + 3 | 7.0 ± 1.4 | 15.4 ± 1.4 | 31.62** |
| | | $F^d$ 2.98** | | |
| Total No. bees: | 1 | 198.5 ± 20.9 | 181.5 ± 14.7 | |
| On colony front | 2 | 248.8 ± 19.2 | 253.5 ± 32.2 | 0.95 |
| | 3 | 102.5 ± 10.5 | 143.7 ± 21.6 | |
| | 1 + 3 | 145.7 ± 12.6 | 161.4 ± 12.7 | 1.49 |
| | | $F^d$ 104.49** | | |
| Flying in front of colony | 1 | 48.8 ± 5.7 | 60.4 ± 6.6 | |
| | 2 | 61.1 ± 6.1 | 69.4 ± 9.8 | 0.18 |
| | 3 | 57.4 ± 7.6 | 60.6 ± 6.8 | |
| | 1 + 3 | 54.6 ± 5.0 | 59.8 ± 4.9 | 2.40 |
| | | $F^d$ 0.44 | | |

[a] Data for each mean are from 12 colonies each tested three times.
[b] Test 1, 6 weeks after empty comb was added to colonies; test 2, 1 week after reversal of treatments; test 3, 6 weeks after reversal of treatments.
[c] $df = 1$; $^{**}P < 0.01$; comparison of treatment; 3.18 CSA vs. 6.36 CSA.
[d] $df = 1$; $^{**}P < 0.01$; comparison of tests; one week after treatment (test 2) vs. six weeks after treatment (tests 1 + 3).

It is not surprising that comb volatiles regulate both foraging and defensive behavior. Established feral colonies with large amounts of empty comb would normally have only comparatively small amounts of stored honey (i.e., early spring, prior to a nectar flow but after brood rearing has resumed). Such colonies would clearly benefit by foraging intensively at high-quality nectar sources if they can be found and at the same time vigorously defending their limited reserve of honey. Normally colonies in nests with less empty comb can be expected to have more stored honey (i.e., after a major honey flow). Such colonies, in defense-eliciting situations not requiring massive responses, may benefit from reduced defense responses which result in fewer bees lost in colony defense. At such times, surviving bees may be more important to a colony than the loss of small amounts of honey from plentiful reserves. Although less empty comb reduces intensity of defense, it does not eliminate it. Presumably, with adequately strong stimulation, such colonies are well able to defend themselves in critical circumstances.

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# OXIDATION OF OLEANOLIC ACID OF *Avicennia officinalis* LEAVES TO OLEANONIC ACID IN THE NATURAL ENVIRONMENT OF SUNDERBAN MANGROVE ECOSYSTEM[1]

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**Abstract**—Oleanolic acid, a major component pentacyclic triterpene in the leaves of *Avicennia officinalis* was found to be oxidized to oleanonic acid in the natural environment of Sunderban mangrove forest.

**Key Words**—*Avicennia officinalis*, pentacyclic triterpene, oleanolic acid, oleanonic acid, oxidation of oleanolic acid, mangrove.

## INTRODUCTION

Mangrove forests are a major supplier of food material to estuarine life. The role of decomposers like fungi and bacteria is well established in mangrove ecosystem (Odum, 1971). Detritus food chains, based on mangrove leaves, have been worked out in considerable detail by Heald (1969) and Heald and Odum (1970). Plant material degradation by fungi produces a variety of organic constituents which, in turn, are exposed to a succession of ecological groups of

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FIG. 1. Structure of oleanolic acid (I) and oleanonic acid (II).

fungi, each organism or group of organisms altering the organic constituents until complete decomposition occurs (Alexander, 1961; Garrett, 1951).

*Avicennia officinalis* plant is one of the dominant plant species of Sunderban mangrove forest in eastern India. During a survey of triterpenoid constituents of leaves in this area, *A. officinalis* was found to contain an appreciable amount of pentacylic triterpenoids, notably, oleanolic acid (I) (Figure 1). In this preliminary study we report the oxidation of oleanolic acid (I) of *A. officinalis* leaves to oleanonic acid (II), in the natural environment of Sunderban mangrove forest.

## METHODS AND MATERIALS

*Study Area.* The study area was on the Prentice Island of Sunderban mangrove forest, situated on the eastern part of India. This island is a virgin unit of Sunderban forest lying between latitude 21.43° and 21.46°N and longitude 88.18° and 88.19°E. The island is criss-crossed by a number of creeks and entertains considerable tidal flow twice daily.

*Field Procedures.* Fresh matured leaves of *A. officinalis* plants were collected from ten different locations, at least 100 m apart, from the banks of the creeks. Leaves were placed in nylon net bags (2.5–5.0 $mm^2$ mesh) and were tied to mangrove roots in ten locations, so that they remained submerged during tidal flow. Leaves were collected from the bags after 45 days. Aliquots of fresh leaf samples were saved for the assay of terpenes.

*Extraction and Isolation of Terpenes.* Fresh and degraded leaves were treated similarly. Leaves were dried, powdered, and stirred for 24 hr with two portions of acetone (Garcia-Alverej et al., 1981). Acetone was evaporated from pooled extracts and evaporated in a vacuum rotary evaporator at 35°. The viscous residue was separated from lipophilic impurities by repeated distribution between light petroleum ether (40–60°C) and MeOH–$H_2O$ (95:5) until the methanolic layer remained colorless (Kraus et al., 1981). The combined methanolic layers were dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure at 40°C.

Preparative TLC of the crude sample was done on 0.25-mm-thick silica gel G, type 60 (E. Merck, Darmstadt, F.R.G.) along with authentic β-amyrin and oleanolic acid in a lane. The solvent system used for development consisted of $CHCl_3$–$CH_3OH$ (95:5). The zones were visualized by iodine vapor. Neutral triterpene bands, corresponding to β-amyrin, and acidic triterpenes, corresponding to oleanolic acid, were scraped off the plate, recovered, and weighed. The purity of neutral and acidic triterpenes was checked by analytical TLC, which was visualized by spraying with Libermann-Burchard spray reagent (Stahl and Jork, 1969).

*Derivatization and GLC of Triterpenes.* Aliquots of authentic and sample neutral triterpenes were silylated (Klebe et al., 1966), using *N,O*-bistrimethylsilylacetamide (Pierce Chemical Company, Rockford, Illinois.). Aliquots of neutral triterpenes were also acetylated (Privette and Nutter, 1967). Triterpene acids were first methylated by diazomethane (Schlenk and Gellerman, 1960). Aliquots of methyl esters were silylated and acetylated. GLC was done on 2% SE-30 and 2% OV-17 liquid phases supported on 80–100 mesh Chromosorb W (HP) and packed into a coiled glass column (2 m × 3 mm). The instrument used was a Pye Unicam gas chromatograph, model GCD with dual FID. Nitrogen carrier gas was used at a flow rate of 60 ml/min. Relative retention times were determined with respect to the acetate and the trimethylsilyl ether of cholesterol.

*MS, PMR, and IR Spectromety* Mass spectra were recorded on a low-resolution AEI mass spectrometer, model MS-30, with an ion voltage of 70 eV. PMR spectra were recorded in $CDCl_3$ on a Varian 90-MHz spectrometer, model EM-390. IR spectra were recorded on Shimadzu spectrophotometer, model IR-408.

## RESULTS AND DISCUSSION

GLC analysis of the neutral triterpene fraction of the fresh leaves of *A. officinalis* revealed the presence of taraxerol (14%), β-amyrin (9%), α-amyrin (21%), and lupeol (56%). GLC analysis of the acidic fraction from fresh leaves showed the presence of oleanolic acid (91%; 52% of total triterpene) and ursolic acid (9%; 5% of total). GLC identification of the triterpenoids was done by comparison of the retention times of the components with those of the authentic standards and also comparison with the retention parameters given in the literature (Wilkomirski and Kasprzyk, 1975; Ikekawa, 1965). The terpene isolated from the degraded leaves had an $R_f$ a little greater than that of oleanolic acid. The $R_f$ was further increased after methylation. The IR spectrum of the methyl ester showed a band at 1705 $cm^{-1}$ (ester carbonyl). The purified methyl ester yielded a single peak on both GLC columns. Acetylation or silylation of the methylated compound did not change the retention time, which was identical to that of methyl oleanonate. The melting point of the methyl ester was 184° which

was identical to that of methyl oleanonate. The terpene acid did not respond to the Libermann-Burchard test.

The PMR spectrum of the methyl ester in $CDCl_3$ showed signals at $\delta$ 5.3 ($-\underline{H}C{=}C\underline{H}-$), $\delta$ 3.62 ($-COOC\underline{H}_3$) $\delta$ 0.78–1.65 ($-C\underline{H}_3$ and $-C\underline{H}_2-$), and $\delta$ 2.35 ($-C\underline{H}_2-CO-$). The mass spectrum of the methyl ester was identical to that of methyl oleanonate (Budzikiewicz et al., 1963). Identical results were obtained with all the samples.

From the above discussion, it has been established conclusively that oleanolic acid (I), a major component of *A. officinalis* leaves has been oxidized to oleanonic acid (II) in the natural environment of Sunderban mangrove forest. Although no attempt has been made in this investigation to explore the cause of oxidation of the 3-$\beta$-hydroxy acid to the 3-keto acid, in all probability, microorganisms are responsible for the transformation.

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# MULTICHEMICAL DEFENSE OF PLANT BUG *Hotea gambiae* (WESTWOOD) (HETEROPTERA: SCUTELLERIDAE)
## Sesquiterpenoids from Abdominal Gland in Larvae

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**Abstract**—Chemical defense in larvae of the plant bug *Hotea gambiae* has been investigated. Results of analyses (GC, GC-MS) on the secretions from the three dorsally situated larval abdominal defense (scent) glands are reported. The secretion from the first abdominal gland consists of a mixture of $C_{10}$ and $C_{15}$ isoprenoids: ($C_{10}$) $\alpha$-pinene, $\beta$-pinene, limonene, $\beta$-phellandrene; ($C_{15}$) $\beta$-caryophyllene, caryophyllene oxide, $\alpha$-humulene, and (the major component) humulene epoxide II. The secretions from the second and third abdominal glands are similar mixtures consisting of (*E*)-2-decenal, (*E*)-4-oxohex-2-enal, and *n*-tridecane together with lesser amounts of (*E*)-2-hexenal, *n*-dodecane, and other materials. Isoprenoid defense is now known from four species of plant bugs (Heteroptera) associated with Malvaceae.

**Key Words**—Scent gland, defensive secretion, *Hotea gambiae* (*H. subfasciata*), Heteroptera, Scutelleridae, Malvaceae, monoterpenoids, $\alpha$-pinene, $\beta$-pinene, limonene, $\beta$-phellandrene, sesquiterpenoids, caryophyllene, caryophyllene oxide, humulene, humulene epoxide, allomones.

## INTRODUCTION

Chemical defense in insect herbivores conforms to no one particular pattern. Some herbivores acquire components of their chemical defense directly by uptake and sequestration of dietary toxins (Blum, 1981; Pasteels et al., 1983). They are often aposematic. Others manufacture their own defensive chemicals, from simple precursors (Blum, 1981). In such cases, chemical defense in the herbivore could be influenced at best only indirectly by the host plant.



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It is interesting that isoprenoid defense, so far found only rarely in Heteroptera, has been found in three species of herbivore which, despite their taxonomic unrelatedness, share a preference for food plants within Malvaceae. They are an alydid (Aldrich et al., 1979), a pyrrhocorid (Everton et al., 1979), and a lygaeid (Olagbemiro and Staddon, 1983; Knight *et al.*, 1984). It is possible that selection for isoprenoid defense within Heteroptera has been favored directly or indirectly by association with Malvaceae. Here we report yet another case of isoprenoid defense from a plant bug associated with Malvaceae.

*Hotea gambiae* (Westwood) is a component of the insect fauna of tropical West Africa (Medler, 1980). Taxonomic opinion is that *H. gambiae* is probably conspecific with *Hotea subfasciata* (Westwood) (Mayné and Ghesquière, 1934; Linnavuori, 1982). The bugs are common on Malvaceae along roads and in clearings in rain and savanna forests (Linnavuori, 1982). In Ghana, they have been recorded as occasional seed pests of okra (Leston, 1980). The work to be reported was carried out on descendents of material originally collected in Senegal. Adults possess two defensive scent glands, one in the thorax (metathoracic scent gland) and one in the abdomen (dorsal abdominal scent gland). Larvae possess a dorsal metameric series of three defensive scent glands in the abdomen.

## METHODS AND MATERIALS

*H. gambiae* was maintained in continuous culture in the laboratory on both seed and seedlings of cotton and wheat (photoperiod 14 hr light, 10 hr dark; daily temperature cycle 18°C night, 28°C day). Body weight data, which can be useful for quantitative considerations of chemical defense, are given in Table 1. Individual weights were recorded as soon as possible after ecdysis, prior to feeding.

Investigation of gland contents by gas chromatography (GC) was carried out using a Varian 1440 gas chromatograph equipped with a flame ionization detector. The 2-m × 2-mm stainless-steel column was packed with 3% OV-225 on 60–80 mesh Gas Chrom Q. Recordings were made using 30 ml nitrogen/min and an injector block temperature of 170°C. After 8 min at 70°C, the oven temperature was programed to 200°C at 6°/min. Sample introduction was either by a solventless open column procedure (Staddon et al., 1979) or, for GC retention tests, by conventional syringe injection of acetone extracts of glandular material.

Gas chromatography–mass spectrometry (GC-MS) was performed in the electron impact (EI) and chemical ionization (CI) modes. For CI GC-MS, methane supplied the reagent gas. The VG 7070E mass spectrometer was operated at 70 eV with the ion source at 180°C, separator 180°C, and 200 μA ionizing current. The separations were achieved with a 2-m × 2-mm glass column packed

TABLE 1. BODY WEIGHT DATA

| Stage | Mean ± SD ($N$)[a] |
|---|---|
| Egg[b] | 1.49 ± 0.06 (13) |
| Larva 1 | 1.45 ± 0.28 (9) |
| Larva 2 | 1.77 ± 0.17 (11) |
| Larva 3 | 5.43 ± 1.05 (4) |
| Larva 4 | 15.55 ± 1.91 (10) |
| Larva 5 female | 47.50 ± 4.10 (12) |
| Adult female | 121.65 ± 15.1 (20) |
| Larva 5 male | 45.75 ± 4.17 (6) |
| Adult male | 109.17 ± 14.93 (23) |

[a] $N$, number of observations.
[b] From weights of individual eggs in a single batch of 13 eggs.

with 3% OV-225 on 100–120 mesh Gas Chrom Q and with ca. 10 ml helium/min. Gland extracts in acetone were injected into the DANI 3800 gas chromatograph under similar conditions to those described above for GC.

Standard chemicals were obtained from a variety of sources: (*E*)-2-hexenal, caryophyllene oxide (Aldrich Chemical Co. Ltd., Gillingham, England); (*E*)-2-octenal, (*E*)-2-decenal, α-pinene, β-pinene, β-phellandrene admixed with α-phellandrene, *n*-tridecane (PPF International, Ashford, England); *n*-dodecane (B.D.H. Chemicals Ltd., Poole, England); *d*-limonene (Sigma London Chemical Co. Ltd., Poole, England); humulene (Brewing Research Foundation, Nutfield, England); and caryophyllene (Koch-Light Laboratories Ltd., Colnbrook, England). Humulene epoxide II was prepared as described by Dauben et al. (1975) by oxidation of humulene using *m*-chloroperoxybenzoic acid (MCPBA). Checks on purity and identity on all standard materials were made by GC-MS and NMR. Proton magnetic resonance (PMR) spectra were obtained in deuterochloroform ($CDCl_3$) using a Brüker 360-MHz instrument. Chemical shifts were measured relative to tetramethylsilane (TMS). Prior purification of sesquiterpenoid samples was achieved by preparative GC using an OV-225 packed column.

## RESULTS

*Morphology.* The metameric series consisting of three dorsal abdominal scent glands is shown in Figure 1. The first (most anterior) gland (dg1) is uniquely divided into paired half-glands by occlusion in the midline. It is without pigment. The second and third glands (dg2, dg3) show no such division, although the orifice in each one takes the form of a pair of ostioles. Their epi-

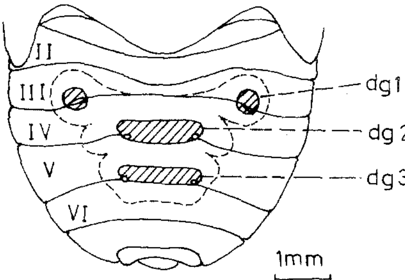


FIG. 1. Topography of abdominal scent gland system in fifth stage *Hotea* larva. dg1, dg2, and dg3: first, second, and third abdominal scent glands. II–VI, abdominal tergites. Limits of sclerotized field surrounding gland openings marked by broken line.

thelium is brick red in color. The openings of all three glands are situated in islands or plates characterized by relatively stiff exocuticle (Figure 1). The exocuticle of the abdominal dorsum, as may be seen by examination of exuviae, is elsewhere relatively soft and flexible.

In *H. gambiae,* the pattern of stretch and opener muscles is similar to that previously described by Henrici (1938) for *Carpocoris*. A reflex emission of the stored secretion usually occurs when larvae are stimulated aggressively by pinching.

All three glands are present and functional in all five larval instars but, of the three, only the first gland continues through the imaginal molt to function in the adult.

*Anterior Gland (dg1) Chemical Analysis.* A typical GC trace is shown in Figure 2a. Similar traces were obtained from dg1 in all five instars. The secretion evidently undergoes little if any change in composition during larval development. The quantitative variations in composition observed may be corre-

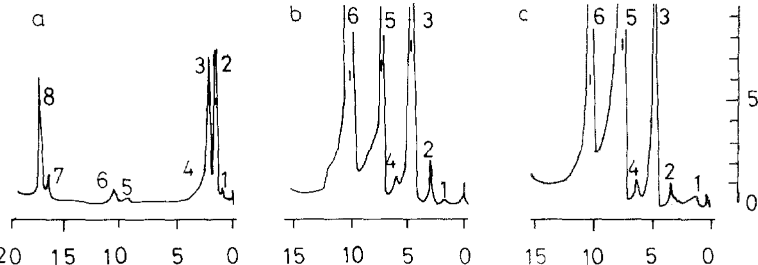


FIG. 2. Gas chromatographic comparisons for dg1, dg2, and dg3. Horizontal scale: time (min) from injection. Range and attenuation $10^{-11} \times 64$. Oven temperature 70° hold for 5 min, and then programed at 6°/min to 200°C. Column 2 m × 2 mm, packed with 3% OV-225 on 60–80 mesh Gas Chrom Q. (a), dg1, instar 4; (b), dg2, instar 3; (c), dg3, instar 3. Traces selected for clarity.

lated with the physiological age of the gland chromatographed. The eight peaks recorded by GC were subsequently identified by GC-MS. Mass spectra, retention times, and peak area data are given in Table 2.

Peaks 1–4 were identified as monoterpenes. Of these components, β-pinene was quantitatively the most important. It contributed ca. 9% to the total peak area observed by GC. α-Pinene (ca. 5%) and limonene (3%) also contrib-

TABLE 2. EI GC-MS AND GC DATA, ANTERIOR ABDOMINAL GLAND (dg1), LARVA

| Peak No. (min)[a] | Identity | Peak area (%)[b] | Mass spectrum m/z (% abundance)[c] |
|---|---|---|---|
| 1 (1.20) | α-Pinene | 4.5 | 93(100), 92(45), 77(23), 91(21), 79(19), 136(M+, 18), 121(13), 80(10), 94(10), 105(8) |
| 2 (2.08) | β-Pinene | 8.7 | 93(100), 41(50), 69(35), 79(21), 77(21), 91(18), 121(13), 94(13), 80(13), 53(13), 67(12), 136(M+,9) |
| 3 (2.56) | Limonene | 3 | 68(100), 93(52), 67(41), 41(24), 79(24), 53(19), 94(20), 121(20), 136(M+,18), 77(12), 107(11), 92(8) |
| 4 (3.32) | β-Phellandrene | 1 | 93(100), 136(M+,44) 43(36), 121(30), 91(25), 77(25), 92(21), 79(18), 68(17), 119(15), 107(9) |
| 5 (16.28) | β-Caryophyllene | 6 | 41(100), 69(88), 93(83), 133(72), 79(54), 91(42), 105(38), 120(38), 161(37), 81(37), 67(27), 148(24), 147(21), 55(20), 189(13), 175(11), 204(M+,10) |
| 6 (17.40) | α-Humulene | 18 | 93(100), 80(39), 121(37), 41(32), 92(19), 67(19), 91(15), 53(14), 107(14), 147(14), 79(14), 204(M+,7) |
| 7 (23.24) | Caryophyllene oxide | 9 | 43(100), 79(58), 93(52), 69(51), 55(38), 95(27), 109(35), 92(33), 68(29), 81(29), 106(29), 105(28), 121(23), 138(17), 149(12), 177(11), 161(9), 187(4), 205(4), 220(M+,3) |
| 8 (24.12) | Humulene epoxide II | 51 | 43(100), 109(93), 138(78), 67(60), 96(58), 41(54), 55(52), 81(38), 68(37), 93(37), 95(35), 82(33), 69(30), 123(24), 53(22), 220 (M+, 3), 152(2) |

[a]Retention time: OV-225, 70°C (5 min) then temperature programed at 6° min to 200°C.
[b]From a single 3rd instar larva.
[c]70 eV; above m/z 40.

uted significantly to the total mixture. β-Phellandrene (1%) was recorded as a quite minor component. All identifications were checked by comparison (mass spectra; retention times on OV-17 and OV-225) with authentic standards. Pinane and limonane were found as expected (GC-MS) in hydrogenated samples.

Peaks 5 and 6 were evidently sesquiterpenes. Initial identification of peak 5 as caryophyllene was based on an EI mass spectrum published for β-caryophyllene (Moshonas and Lund, 1970) and subsequently confirmed by comparison with an authentic sample of purified material (EI mass spectrum; retention times on OV-17 and OV-225). Attempts were made to compare products of hydrogenation in *Hotea* with authentic caryophyllanes (see Kepner and Maarse, 1972), but peak intensities of reduction products obtained from the *Hotea* sample were too weak for analysis.

From accurate high-resolution mass measurement, $M^{+\bullet}$ for peak 7 and $M^{+\bullet}$ for peak 8 were found to be *m/z* 220.1826, indicating a molecular formula of $C_{15}H_{24}O$ for both peaks (*m/z* calculated for $C_{15}H_{24}O$, 220.1827).

Peak 7 was identified as caryophyllene oxide from mass spectral data (EI and CI) and GC retention tests (OV-17, OV-225) in comparison with authentic caryophyllene oxide (Aldrich). The EI mass spectrum of the caryophyllene oxide from *Hotea* matched that published by Hashizume and Sakata (1970) for (−)-β-caryophyllene oxide from peppermint oil.

Peak 8 was similarly identified as humulene epoxide II by comparison of mass spectral data (EI, CI) and retention tests (OV-17, OV-225) with humulene epoxide II prepared by oxidation from α-humulene (Dauben et al., 1975).

Preparation of humulene epoxide II by oxidation from α-humulene by the method of Dauben et al. (1975) is likely to produce some humulene epoxide I, in addition to the main component, but not humulene epoxide III. While the infrared spectrum from our synthetic material matched that published for humulene epoxide II (Damodaran and Dev, 1968), the presence of a small quantity of humulene epoxide I (ca. 3% eluting before the main peak) was indicated by the GC and EI GC-MS data obtained from the synthetic material. The mass spectrum of this minor component showed a base peak at *m/z* 93 and major ions at *m/z* 80, 121, 107, 41, 205, 220 ($M^{+\bullet}$), matching that of humulene epoxide I (Dr. T.L. Peppard, Brewing Research Foundation, personal communication).

Caryophyllene (6%) and humulene (18%) contributed significantly to the total peak area response recorded by GC. Caryophyllene oxide (9%) was a major component, but humulene epoxide (51%) contributed most to the total peak area response.

*Middle and Posterior Gland (dg2, dg3) Chemical Analysis.* Typical GC traces are shown in Figure 2 B and C. The two glands evidently produce similar secretions. Quantitative but not qualitative variations were observed in samples chromatographed from dg2 and dg3 from all five instars. Mass spectra, retention times, and peak area data for the five peaks recorded by GC are given in

TABLE 3. EI GC-MS AND GC DATA, MIDDLE AND POSTERIOR ABDOMINAL GLANDS (dg2 + dg3), LARVA

| Peak No. (min)[a] | Identity | Peak area (% dg2; % dg3)[b] | Mass spectrum[3] m/z (% abundance)[c] |
|---|---|---|---|
| 1 (3.29) | (*E*)-Hex-2-enal | 0.2; 1.2 | 41(100), 55(83), 69(62), 42(62), 57(52), 83(41), 43(26), 70(22), 98(M+,20), 97(13), 80(11) |
| 2 (4.52) | *n*-Dodecane | 0.2; 2.8 | 57(100), 43(86), 71(56), 85(31), 56(16), 170(M+,12), 55(11), 70(11), 84(7), 99(7), 113(6), 98(5), 127(2), 141(1) |
| 3 (8.56) | *n*-Tridecane | 59; 43 | 57(100), 43(80), 71(58), 85(38), 56(13), 55(13), 70(12), 184(M+,10), 42(10), 99(10), 98(8), 113(8), 127(7), 112(5) |
| 5 (12.16) | (*E*)-4-Oxohex-2-enal | 14; 29 | 83(100), 55(43), 112(M+,24), 84(17), 57(17), 56(7), 54(4), 55(4), 69(2), 97(1) |
| 6 (16.40) | (*E*)-Dec-2-enal | 26; 21 | 43(100), 70(84), 41(75), 55(72), 57(55), 83(47), 69(39), 56(35), 42(24), 98(24), 97(19), 110(15), 136(8), 121(5) |

[a] Retention time: OV-225, 70°C (5 min), then temperature programed at 6°/min to 200°C.
[b] From a single 3rd instar larva.
[c] 70 eV; above *m/z* 40.

Table 3. The first two peaks, (*E*)-2-hexenal (dg2, 0.2%; dg3, 1.2%) and *n*-dodecane (dg2, 0.2%; dg3, 2.8%), were quite minor components. Peak 3, *n*-tridecane (dg2, 59%; dg3, 43%), was the major component of these secretions. Peaks 4 and 5, (*E*)-4-oxohex-2-enal (dg2, 14%; dg3, 29%) and (*E*)-dec-2-enal (dg2, 26%; dg3, 21%), respectively, also made significant contributions to the peak area total (GC).

In all cases, confirmation of identity was obtained by comparison of EI mass spectra and retention times (OV-17, OV-225) of *Hotea* and authentic materials. A sample of (*E*)-4-oxohex-2-enal was prepared from (*E*)-2-hexenal [5mg (*E*)-2-hexenal stirred with 5.5 mg $SeO_2$ in ethanol for 2 hr at room temperature yielded a usable quantity of (*E*)-4-oxohex-2-enal for GC-MS; EI mass spectrum of (*E*)-4-oxohex-2-enal published by Gilby and Waterhouse, 1965]. The *m/z* 154 $M^{+\bullet}$ of (*E*)-2-decenal was revealed by CI GC-MS using methane as reagent gas.

In a subsequent study, two additional quite minor components were identified from their EI mass spectra as (*E*)-2-octenal and *n*-pentadecane, respectively.

## DISCUSSION

A possible link within Heteroptera between the production of isoprenoids for defense and herbivory on food plants within Malvaceae is indicated by the facts now known on chemical defense in heteropteran herbivores (Staddon, 1979; Blum, 1981; Pasteels et al., 1983). However, there is wide variation in chemical defense in the four isoprenoid producers associated with Malvaceae. In *Hotea*, isoprenoids dominate the secretion from the first larval abdominal gland and elsewhere in the scent gland system occur only to a limited extent (the metathoracic scent gland in adults, Hamilton et al., 1985). In *Dysdercus* (Pyrrhocoridae) the production of linalool is confined to the metathoracic scent gland in adults (Everton *et al.*, 1979). $(Z,E)$-$\alpha$-Farnesene, which dominates the secretion from the metathoracic scent gland in mature adults of the lygaeid *Oxycarenus hyalinipennis*, has not been found in the larva (Olagbemiro and Staddon, 1983; Staddon and Olagbemiro, 1984; Knight et al., 1984). Among other insect herbivores which live high on their food plants, isoprenoid defense has been recorded in *Papilio* larvae from Umbelliferae (Honda, 1981) and, within Heteroptera, in a mirid from oak (Hanssen and Jacob, 1982).

The occurrence of isoprenoid defense in Heteroptera showing a preference for Malvaceae could be associated with the fact that species within Malvaceae (*Gossypium, Hibiscus*) possess extrafluoral nectaries (Bentley, 1977). The secretions from floral and extrafloral nectaries are attractive to ants, parasitoids, and some other insect predators of insect herbivores (Bentley, 1977; Price et al., 1980). Nectaried plants, through their attractiveness for ants, could be one means of conditioning chemical defense in insect herbivores.

Caryophyllene oxide from natural sources probably enters into ecological interactions in a diversity of different ways. Antifungal properties may determine its presence in tropical legumes (Arrhenius and Langenheim, 1983; Hubbell et al., 1983). Its presence in chemical defense in *Papilio* larvae may be associated with a particular repellency to ants (Honda, 1981). We are not aware of similar studies on humulene epoxide II. Common requirements for defense presumably explain the occurrence of isoprenoids in *Hotea* larvae (this paper) and their host plants within Malvaceae (Pomonis et al., 1980).

We have not detected in cotton seed the sesquiterpenoids represented in dg1 of *Hotea* larvae (EI GC-MS of cotton seed). This indicates that *Hotea* larvae manufacture their own sesquiterpenoids for defense.

The need for isoprenoid defense in *Hotea* larvae, in addition to the more usual carbonyl defense, perhaps explains the retention of a gland (dg1) which has suffered widespread reduction in Pentatomoidea (Dupuis, 1947a, b).

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# EXOCRINE SECRETIONS OF BEES
## VI. Unsaturated Ketones and Aliphatic Esters in the Dufour's Gland Secretion of *Dufourea novaeangliae* (Hymenoptera: Halictidae)

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**Abstract**—The volatile components of the Dufour's gland extracts of *Dufourea novaeangliae* were analyzed by gas chromatography-mass spectroscopy. The following were identified: a series of five bis-homologous unsaturated ketones ranging from $C_{10}$ to $C_{18}$, a series of nine bis-homologous hexanoates ranging from $C_8$ to $C_{24}$, a series of three bis-homologous octanoates ranging from $C_8$ to $C_{12}$, and hexanoic, hexadecanoic, and octadecanoic acids. The ketones are all new natural products reported for bees. Several of these unsaturated ketones were also identified in extracts of the provision masses from their nest cells. Male mandibular gland extracts contained citral.

**Key Words**—Bees, Hymenoptera, Halictidae, *Dufourea,* Dufour's gland, mandibular gland, exocrine products, unsaturated ketones, aliphatic esters, citral, nest cell provisions.

## INTRODUCTION

The Halictidae is a large, cosmopolitan family of bees that includes typical soil-nesting species as well as wood-dwelling and cleptoparasitic ones. It is divided

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into three subfamilies: the Halictinae or "sweat bees," the Nomiinae, and the Dufoureinae (Michener, 1974). The Dufoureinae is a relatively small group of nonsocial halictids, frequently specialists for pollen on specific genera of plants (oligolectic) and are best represented in the Holarctic region. There are approximately 70 species of *Dufourea* in the United States, primarily in the West (Hurd, 1979).

The Dufour's glands of approximately 40 species of Halictinae and four species of Nomiinae have been analyzed (Andersson et al., 1966; Bergström, 1974; Hefetz et al., 1978; Bergström and Tengö, 1979; Duffield et al., 1981, 1982; Johansson et al., 1982). In contrast, a Dufour's gland extract of only one species of Dufoureinae, *Dufourea marginata,* has previously been analyzed (Cane, 1983a). It contains $C_{23}$, $C_{25}$, and $C_{27}$ alkanes, a $C_{27}$ alkene, octadecyl hexanoate, octyl octadecanoate, octyl eicosanoate, and two compounds of unknown structure.

In this report we identify a series of esters and unsaturated ketones from the Dufour's gland secretions of another species of *Dufourea, Dufourea novaeangliae.* Mandibular gland components, not previously reported in the Dufoureinae, were also identified. Male mandibular glands, but not those of females, contain geranial and neral.

## METHODS AND MATERIALS

During July and August of 1980 and 1981, *Dufourea novaeangliae* were collected at flowers of *Pontederia cordata* (Pickerelweed) at Fairhaven Beach State Park, Fairhaven, New York. Individual specimens were placed in glass shell vials and stored on ice. The Dufour's glands were excised under water and extracted in methylene chloride. Three extracts were prepared of approximately 30 glands each. Extracts of female heads were prepared similarly with 30 heads per vial of methylene chloride. In addition, male and female heads were removed with forceps, and mandibular glands were excised, pooled, and extracted: 10 pairs from males and 20 from females.

Several nests were excavated. Larval cell provisions (eight pollen–nectar balls) were removed and extracted with methylene chloride. Cell walls were scraped free of soil and extracted with methylene chloride.

Extracts were analyzed on a Finnigan 3200 computerized gas chromatograph–mass spectrometer (GC-MS) utilizing a 2-m × 1-mm glass column, containing 3% OV-17 on Supelcoport 60–80 mesh, temperature programed from 60°C to 300°C at 10°C/min, and a 10% SP-1000 column programed from 50°C to 200°C at 10°C/min. Individual compounds were identified by comparison of their mass spectra and retention times with those of standard compounds.

Unsaturated ketones were prepared by the addition of the appropriate alkyl lithium (Gilman et al., 1949) to acrolein, followed by oxidation of the allylic alcohol (Mancera et al., 1953) to the corresponding ketone.

## RESULTS

The Dufour's gland extracts of *D. novaeangliae* exhibit three homologous series eluting over a 200°C range on an OV-17 column. Two of these series showed the characteristic $RCO^+$, $RCO_2H_2^+$, and alkene peaks of straight-chain esters (Fernandes et al., 1981) corresponding to esters of hexanoic and octanoic acids with alcohols ranging from $C_8$ to $C_{24}$. This mixture of isomeric esters was simpler than those found in andrenid (*Andrena*) (Fernandes et al., 1981) or anthophorid bees (*Svastra*) (Duffield et al., 1984a). The molecular weights of these ester mixtures ranged from 228 ($C_8$–$C_6$) to 452 ($C_{24}$–$C_6$) with the higher-molecular-weight hexanoates predominating (Figure 1).

The third homologous series (Figure 1) exhibited base peaks at *m/z* 55 with

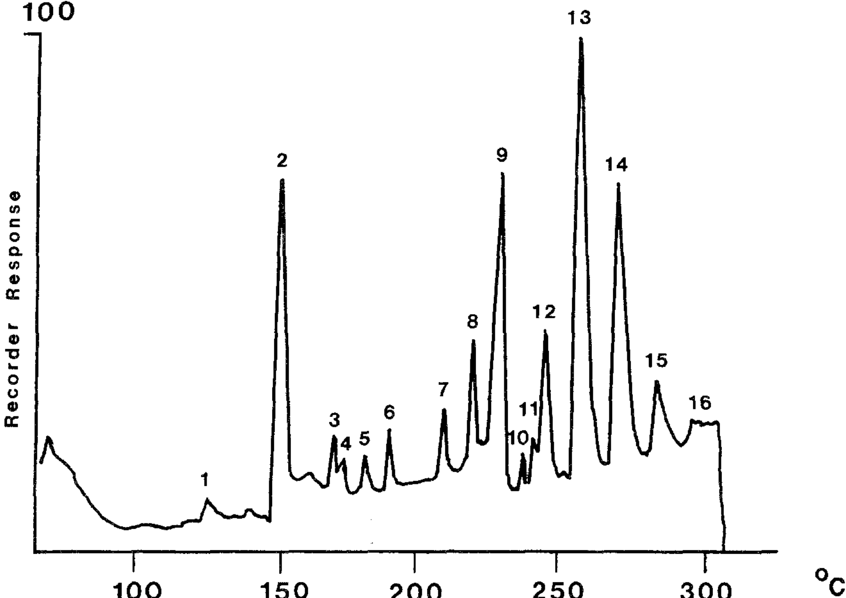


FIG. 1. Gas chromatogram of *Dufourea novaeangliae* Dufour's gland secretion. Peaks correspond to: (1) hexanoic acid (mol. wt. 116); (2) 1-dodecen-3-one (mol. wt. 182); (3) octyl hexanoate (mol. wt. 228); (4) 1-tetradecen-3-one (mol. wt. 210); (5) unknown enone ($C_{15}$?); (6) decyl hexanoate and octyl octanoate (mol. wt. 256); (7) dodecyl hexanoate and decyl octanoate (mol. wt. 284); (8) 1-octadecen-3-one (mol. wt. 266); (9) tetradecyl hexanoate and dodecyl octanoate (mol. wt. 312); (10) hexadecanoic acid (mol. wt. 256); (11) octadecanoic acid (mol. wt. 284) and hexadecyl hexanoate (mol. wt. 340); (12) $C_{25}$ alkane (mol. wt. 352); (13) octadecyl hexanoate (mol. wt. 368); (14) eicosyl hexanoate (mol. wt. 396); (15) docosyl hexanoate (mol. wt. 424); and (16) tetracosyl hexanoate (mol. wt. 452). 1-Decen-3-one (mol. wt. 154) and 1-hexadecen-3-one (mol. wt. 238) were present in smaller amounts and are not labeled here. Drawing from the original.

TABLE 1. UNSATURATED KETONES IDENTIFIED FROM DUFOUR'S GLAND SECRETIONS OF *Dufourea novaeangliae*

| Ketone | Mol wt | Elution temperature (°C) | m/z (%) |
|---|---|---|---|
| 1-Decen-3-one | 154 | 125 | 125(10), 111(5), 97(8), 83(15), 70(88), 55(100), 43(18), 41(60) |
| 1-Dodecen-3-one | 182 | 148 | 153(1), 139(2), 126(4), 111(5), 109(10), 97(5), 83(15), 70(77), 55(100), 43(10), 41(42) |
| 1-Tetradecen-3-one | 210 | 173 | 181(6), 139(8), 126(5), 111(8), 97(13), 83(20), 70(95), 55(100), 43(37), 41(56) |
| 1-Hexadecen-3-one | 238 | 205 | 209(5), 139(8), 126(5), 111(8), 97(13), 83(21), 70(85), 55(100), 43(45), 41(60) |
| 1-Octadecen-3-one | 266 | 230 | 209(4), 153(4), 139(4), 126(4), 111(6), 97(8), 83(16), 70(66), 55(100), 43(20), 41(28) |

large peaks at *m/z* 70, 41, 83, and 97. The series consisted of five compounds, $C_{10}$, $C_{12}$, $C_{14}$, $C_{16}$, and $C_{18}$ and appeared to be unsaturated ketones with the $C_{12}$ predominating. Comparison with a published spectrum of 1-tetradecen-3-one isolated from extracts of *Schedorhinotermes putorius* soldier termites (Quennedey et al., 1973) suggested that these were 1-en-3-one compounds. Comparison with spectra obtained from soldier head extracts of *S. lamanianus* indicated that these were indeed 1-en-3-one compounds. The mass spectra (see Table 1) and retention times of synthetic samples were identical to those of the natural material from the bees.

Chemical analyses of the nest cell provisions of *D. novaeangliae* showed the presence of 1-dodecen-3-one, 1-tetradecen-3-one, methyl hexadecanoate, and ethyl hexadecanoate. None of the esters found in the Dufour's gland extracts were detected. No volatile compounds were detected in extracts of cell walls.

Analyses of male head extracts showed two components identified as geranial and neral. Extracts of isolated mandibular glands showed these to be the source of the aldehydes. Although citral was readily identified in extracts of a smaller number of male heads, no citral was detected in female head extracts.

## DISCUSSION

The Dufour's gland chemistry has been investigated in all three subfamilies of the Halictidae. Dufour's glands from species of Halictinae contain primarily

hydrocarbons and saturated and unsaturated macrocyclic lactones (Andersson et al., 1966; Bergström, 1974; Hefetz et al., 1978; Bergström and Tengö, 1979; Duffield et al., 1981; Johansson et al., 1982) as well as traces of isopentenyl esters (branched $C_5$ alkenols and fatty acids) (Duffield et al., 1981). The four species analyzed in the Nomiinae contain hydrocarbons, a homologous series of saturated macrocyclic lactones, and a series of isopentenyl esters, the last representing major components in each secretion (Duffield et al., 1982).

The Dufour's gland secretion of *Dufourea marginata* contains hydrocarbons ($C_{23}$, $C_{25}$, $C_{27}$ alkanes, a $C_{27}$ alkene), esters (octadecyl hexanoate, octyl octadecanoate, and octyl eicosanoate) and two "undetermined acetogenins" (Cane, 1983a). Cane presented no spectral information for the two unknowns or the other compounds. The Dufour's gland extract of *Dufourea novaeangliae* appears to be more complex chemically. There are two compounds in common with *D. marginata:* octadecyl hexanoate and the $C_{25}$ alkane. Both of these are common insect natural products. For further discussion of the possible phylogenetic relationship of *Dufourea* to andrenid and melittid bees, see Cane (1983b).

The aliphatic esters (nine hexanoates and three octanoates) in extracts of *D. novaeangliae* also occur in the Dufour's gland extracts of species of *Andrena* (Andrenidae) (Fernandes et al., 1981). All three octanoates (octyl, decyl, dodecyl) have been reported in Dufour's gland extracts of *Svastra obliqua obliqua* (Anthophoridae) (Duffield et al., 1984a). Decyl octanoate has recently been reported as a minor caste-specific component of the queen honeybee (*Apis mellifera*) sting apparatus (Blum et al., 1983) and in the Dufour's gland of *Centris analis* (Cane and Brooks, 1983). Octadecyl hexanoate, eicosyl hexanoate, docosyl hexanoate, and tetradecyl hexanoate have been reported in various combinations in seven species of *Perdita* (Andrenidae) (Cane, 1983b).

Simple vinyl ketones were first identified as insect natural products by Quennedey et al. (1973) from the frontal gland secretions of the termite *Schedorhinotermes putorius.* They included 1-tetradecen-3-one and 1-hexadecen-3-one. Subsequently, Prestwich et al. (1975) identified these two compounds, as well as 1-dodecen-3-one, from another species of *Schedorhinotermes.* These three vinyl ketones, as well as 1-decen-3-one and 1-octadecen-3-one, are found in the Dufour's gland extracts of *D. novaeangliae.* The latter two are new insect natural products. None of the five compounds has previously been identified in the Apoidea.

Chemical analyses of the pollen–nectar provisions removed from the nest cells of *D. novaeangliae* gave unexpected results. The presence of major Dufour's gland components in cell provisions has previously been reported by Duffield et al. (1981) for the halictine bee, *Augochlora pura.* The major vinyl ketones, 1-dodecen-3-one and 1-tetradecen-3-one, are present in the provisions of *D. novaeangliae.* Since the sample was small (eight provision masses), it is not surprising that the minor Dufour's gland components were not detected. What is striking is that none of the 12 esters (hexanoates and octanoates) was de-

tected, including octadecyl hexanoate, which is the major compound of the secretion.

The other two esters detected in the provisions of *D. novaeangliae,* methyl hexadecanoate and ethyl hexadecanoate, are not present in the Dufour's gland extracts. These may be contributed by pollen or nectar from *Pontederia.*

It is interesting to note that in *Schedorhinotermes* extracts, the vinyl ketones are also the dominant compounds. There, each vinyl ketone is accompanied by traces of the corresponding saturated ketone and dienone. When examined on a 10% SP-1000 column, Dufour's gland extracts of *D. novaeangliae* showed two peaks where the 1-dodecen-3-one had eluted previously. The second peak exhibited a molecular ion at *m/z* 180(0.1), a base peak at 55, and important fragments at 41(30), 70(70), 83(20), 97(5), 109(18), 123(2), 133(2), 139(1), 151(1), and 165(0.5), in accord with published spectra of dienones (Prestwich et al., 1975).

The chemical data for the larval provisions of *D. novaeangliae* pose several questions pertinent to the chemical ecology of bees. Are Dufour's gland secretions modified after they are added to the provisions? What is the source of the other volatile components (methyl and ethyl hexadecanoate) of the provisions? One function of the Dufour's gland secretions in the halictid bees is to provide chemicals to form a waxlike, waterproof lining for the brood cells (Michener, 1974). The cell linings of *Augochlora pura* (Duffield et al., 1981) and *Evylaeus albipes* (Cane, 1981) have been shown to contain the same major constituents as the Dufour's gland. Similar evidence has been reported for species in the Andrenidae (Cane, 1981), Anthophoridae (Norden et al., 1980), and the Colletidae (Hefetz et al., 1979). Brooks and Cane (1984) have shown that the halictid, *Halictus hesperus,* uses Dufour's gland secretion to line the nest entrance turret.

This report of citral in the male mandibular gland secretion of *D. novaeangliae* is the first such identification in the Halictidae. Geranial and neral (citral) are common mandibular gland components in other Apoidea. They have been identified in exudates of species of Andrenidae—*Andrena* (Tengö and Bergström, 1976), and *Panurginus* (Duffield et al., 1983; Wheeler et al., 1984); Colletidae—*Colletes* (Bergström and Tengö, 1978; Hefetz et al., 1979), and *Hylaeus* (Blum and Bohart, 1972; Bergström and Tengö, 1973; Duffield et al., 1980); Anthophoridae—*Centris* (only neral present) (Vinson et al., 1982); and Apidae—*Trigona* (Blum et al., 1970; Crewe and Fletcher, 1976), and *Lestrimelitta* (Blum, 1966).

The function of the mandibular glands in *Dufourea* is unknown. In other bees (reviewed by Duffield et al., 1984b), the secretion is a defensive allomone or an attractive pheromone that marks nest aggregations and male "sleeping" aggregations. Male mandibular gland secretions are also used to mark territories, leks, and patrol routes and to mark females during mating, which in cleptoparasitic bees presumably facilitates female entry into host nests. Another

suggested role for the mandibular gland secretions of solitary bees is for nest cell disinfection (Cane et al., 1983). The presence of citral in male but not female glands in *D. novaeangliae* suggests a function in mating or male-male interactions, although Kukuk et al. (1984) did not observe such a function in their behavioral study.

The glands and their secretions may also have taxonomic significance. The presence of well-developed mandibular glands and the ability to produce citral appears to be primitive in bees, and the loss of both in the Halictinae and Nomiinae is thus a synapomorphy uniting these two subfamilies, but not to the Dufoureinae which retains the plesiomorphic state. Similarly (as noted by Cane, 1983), the Dufour's glands secretions of the Dufoureinae lack the saturated and unsaturated macrocyclic lactones which comprise a strong synapomorphy of the Halictinae and Nomiinae (Duffield et al,. 1981, 1982). Instead, the bis-homologous series of vinyl ketones uniquely characterize *Dufourea novaeangilae* and do not provide evidence linking the Dufoureinae to any other apoid taxon. These new data support systemic decisions based on morphological characters that place the Dufoureinae at the base of the halictid lineage, quite well separated from the closely linked Nomiinae and Halictinae. Indeed, there are no glandular or chemical synapomorphies that would unite the Dufoureinae with the other two subfamilies in a common family.

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# STUDIES ON POSSIBLE ROLE OF CATECHOLAMINES IN REGULATION OF SEX PHEROMONE GLAND ACTIVITY IN AMERICAN DOG TICK, *Dermacentor veriabilis* (Say)[1]

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**Abstract**—Administered monoamines affected sex pheromone activity in the foveal glands of the tick, *Dermacentor variabilis* (Say). Flooding the tissues of the female tick with reserpine, α-methyl-*m*-tyrosine methyl ester hydrochloride, and pilocarpine prior to feeding led to reductions in female sex attractant activity during engorgement. Similar treatments with cyclic AMP, dopamine, serotonin, 6-hydroxydopamine, and acetylcholine had no apparent effects on the attractiveness of feeding females. Assays (by gas chromatography) demonstrated substantial reductions in 2,6-dichlorophenol content following treatment with α-methyl-*m*-tyrosine methyl ester, pilocarpine, and, in most cases, with reserpine. Reserpine was effective only when administered in near-lethal concentrations to unfed females. In contrast, treatment with dopamine led to elevated 2,6-dichlorophenol content in most trials. X-ray microanalysis corroborated the evidence with reserpine and dopamine. These and other findings reported elsewhere implicate monoamines, presumably catecholamines, in the regulation of sex pheromone secretion in this species. The significance of these findings for understanding the physiological mechanisms involved in the regulation of sex pheromone secretion and biosynthesis is discussed.

**Key Words**—*Dermacentor variabilis*, tick, Acarina, Ixodidae, catecholamines, monoamines, pheromone secretion.

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## INTRODUCTION

Much has been learned about the biology of tick pheromones following Berger's (1972) discovery of 2,6-dichlorophenol as the sex pheromone of *Amblyomma americanum*. Recent reviews (Leahy, 1979; Sonenshine et al., 1982a) describe the chemical characteristics, periods of activity, species specificity, and glandular sources of these biologically active molecules, but the physiological processes regulating sex pheromone activity in hard ticks remain unknown. In many species, the foveal glands, located in the mid-dorsal region of the tick's body, store and secrete the common sex pheromone 2,6-dichlorophenol (Sonenshine et al., 1977). These glands are innervated by the foveal nerves (Sonenshine et al., 1981). Clearly, neurosecretory substance or neurotransmitter excitation of sex pheromone secretion represents one hypothesis for neural control of sex pheromone secretion that can be considered.

Catecholaminergic neural control of tick salivary gland secretion has been postulated by several workers (Kaufman and Sauer, 1982, review). Copious secretion is elicited, in vivo as well as in vitro, by treatment with dopamine, epinephrine, and norepinephrine, suggesting the presence of catecholaminergic receptor sites in these glands. Similarly, pilocarpine has been found to stimulate tick salivary secretion when administered in vivo. Pilocarpine may be acting on the cholinergic receptors on the catecholamine neurons that innervate these glands. Thus, both catecholaminergic and cholinergic neurotransmitter systems may participate in the control of salivary gland secretion. Acetylcholine, acetylcholinesterase, dopamine, and norepinephrine have all been demonstrated to occur in tick synganglia (Binnington and Obenchain, 1982). Octopamine, found in some insects, may also be present in ticks, but no evidence of its ability to stimulate salivary secretion in vivo was found (Needham and Pannabecker, 1983). Thus, several possible pathways for control of tick glandular secretion exist.

This study investigates the effects of a variety of putative neurotransmitters, selected antagonists, and other pharmacologically active compounds on the functioning of the sex pheromone glands in the American dog tick, *Dermacentor variabilis* (Say). The implications of the findings for understanding catecholamine regulation of tick sex pheromone gland activity are discussed.

## METHODS AND MATERIALS

*Ticks*. *Dermacentor variabilis* was colonized with specimens obtained near Montpelier and Ashland, in central Virginia. Ticks were reared and stored for experiments as described in previous reports (Sonenshine et al., 1977). The mean weight of the laboratory-reared unfed females used in this study was 6.13 ± 0.71 mg ($N$ = 22), and of the females fed 7 days, 99.02 ± 36.32 mg ($N$ = 72).

*Treatments*. To test the effects of the various compounds under study, the tissues of the ticks were flooded with solutions of these materials by injection of large volumes of fluid relative to the body weights of these animals. Five microliters of a $10^{-3}$ M solution of dopamine, 6-hydroxydopamine (6-OH-dopamine) (as the hydrochloride), cyclic AMP, acetylcholine, pilocarpine, serotonin, reserpine (Sigma Chemical Co., St. Louis, Missouri) or DL-$\alpha$-methyl-*m*-tyrosine methyl ester hydrochloride (98% pure, MTME, a gift from P. Papavasiliou, Veterans Hospital, Hampton, Virginia) was administered to *D. variabilis* females. Ticks apparently tolerate these dosages, which exceed typical physiological concentrations, as demonstrated by Kaufman (1978).

The compounds were injected into the posterior body area of unfed females, two to four weeks postemergence, with a 50-$\mu$l Hamilton syringe and a 30-gauge needle (Analabs Inc., North Haven, Connecticut). Delaying withdrawal of the needle for ca. 10–12 min minimized leakage. Mortality varied from ca. 10% to 22% except in the case of reserpine, in which mortality exceeded 70% in most treatments. Twenty-four hours after these treatments, surviving individuals were allowed to feed on laboratory rabbits, *Oryctolagus cuniculus*. Injections of reserpine were also made into partially fed females 24 hr before they were forcibly detached on the 7th day of feeding. Controls were injected with Shen's solution (SS) (Oliver, 1972), propylene glycol–Shen's (PGSS, see below) or were untreated; in some cases, 1% saline was used. Mortality with the controls was less than 10%, except with the PGSS controls for the reserpine tests.

The pharmacologically active compounds were aqueous solutions, except the reserpine solution which was prepared by dissolving the solid in 10 $\mu$l of 10% citric acid and diluting to 10 ml with 10% propylene glycol–Shen's solution (v/v). Mortality with this latter solvent alone was 39%; when reserpine was included, ca. 70%. Attempts to administer reserpine to tick-infested rabbits by intramuscular inoculation of the injectable formulation Serapsil® (Ciba Pharmaceutical Co., Summit, New Jersey), proved severely injurious or lethal to the animals and had to be discontinued before the ticks had commenced engorging. Instead, Serapsil was administered directly to unfed female ticks as described above.

In addition to introduction of reserpine by direct inoculation, one sample of unfed females was treated with a solution of $5 \times 10^{-2}$M reserpine dissolved in 2 $\mu$l of dimethyl sulfoxide (DMSO) (Fisher Scientific Co., Silver Springs, Maryland, spectranalyzed) applied topically to the posterior alloscutal area of each female. The alloscutal surface was washed with 1–2 $\mu$l hexane prior to treatment to remove any 2,6-dichlorophenol on the body surface. The ticks were restrained (with tape) for 4–6 hr to allow sufficient time for penetration of the test compound. Excess fluid was removed before the ticks were released and allowed to feed on a rabbit host. Controls received DMSO only.

Individual ticks from each experimental group were weighed prior to anal-

ysis and compared to the weight of tick controls (Shen's solution, 1% saline, and untreated), using a Sartorius model 2462 semimicrobalance.

*Bioassay.* Bioassays to determine treated female secretion of sex attractant pheromone were described previously by Sonenshine et al. (1977) and Khalil et al. (1981). Females used in these tests received the same pharmacologically active compounds in the same concentrations described above. The bioassays were done on rabbits with ticks attached to these animals. Each female was assayed with five males, and each male was allowed three trials. Males that proved unsuitable or attached to the host after a mating attempt were discarded. Observations were made to determine whether the males oriented and, having contacted the females, whether the males would attempt copulation. Controls were assayed in the same manner but on separate hosts. The frequency of positive responses divided by the number of mating attempts gave the percentage of positive responses for each test.

Bioassays were also done to determine the ability of a treated female to attract sexually active males at a distance. All other females within a radius of 10 cm from the test specimens were removed to exclude attraction from other sources. Sexually active males were placed at distances from the treated female of 3, 2, or 1 cm, or on the female, and the orientation responses of each male was recorded. Each female was tested with five males; at least six females were used for each treatment. This assay provides an opportunity to discriminate intensity of pheromone output. Presumably, females secreting vigorously would be attractive at a considerable distance, and most males would orient to them when they approached to within 2–3 cm. In contrast, females with diminished secretory activity would be expected to be less attractive, and most males would orient only when very close or in direct contact.

*Gas Chromatography.* Partially fed ticks that were previously treated as described above were placed in ampoules containing cold (−60°C) spectrophotometric grade, double-distilled hexane (Krackler Chemical Co., Albany, New York). The ampoules were flame sealed and shipped to the Department of Chemistry, State University of New York, Syracuse, New York, for chemical studies. Samples damaged in transit were discarded. The remaining sealed ampoules were subjected to repeated cycles of freezing in liquid nitrogen, thawing, and sonication with a Bransonic II ultrasonic probe (Branson Sonic Power Company, Danbury, Connecticut) (Sonenshine et al., 1976). The hexane was decanted from the residue, and the procedure was repeated twice with double-distilled pentane.

Two different methods for extraction and assay of the pheromone were used. In method 1, essentially the same as that described by Sonenshine et al. (1984), the hexane–pentane solution was concentrated to about 5 ml by distillation through a packed column, and this solution was cooled and extracted three times with ice-cold 3% NaOH. The cold NaOH extract was acidified with ice-cold 4% HCl to pH 2, and the acidified solution was extracted three times with pentane

and once with diethyl ether. The pentane and ether solutions were washed with water, dried over $Na_2SO_4$, and concentrated to about 1 ml by distillation through a packed column. The concentrated pentane solution was fractionated by gas chromatography (GC). Aliquots of tick material were injected on a Varian model 2740 gas chromatograph. The three individual GC fractions and a total (unfractionated) GC collection were rinsed from the collection capillaries (Brownlee and Silverstein, 1968) with 100 $\mu$l hexane. Fractionation (flame ionization detector) was carried out as follows: 3.1 m × 4 mm ID glass column, 5% OV-101 on Chromosorb G 50–80 mesh; the carrier gas was nitrogen at a flow rate of 60 ml/min; temperature was programed from 110° to 200°C. Under these conditions, 2,6-dichlorophenol (2,6-DCP) eluted in 21 min. The presence of 2,6-DCP in fraction 2 (collected between 18 and 24 min) was verified by GC assay of the same fraction with an electron capture detector (3.1 m × 1.5 mm ID glass column with 4% FFAP on Chromosorb G 80–100 mesh, nitrogen at 20 ml/min, temperature 170°C). Coinjection with an authentic sample gave a single sharp peak. Capillary column chromatography (flame ionization detector) of fraction 2 also gave a single sharp peak, as did coinjection with an authentic sample (Varian 50 m × 0.21 mm ID fused silica FFAP column).

In method 2, the crude extracts were passed through a 6 cm × 6 mm ID glass column packed with Florisil (Waters Assoc., Milford, Massachusetts) and eluted as described by Sonenshine et al. (1982b). Recovery with this method was ca. 95%. Assay of the eluates was done by GC as described above.

*Histochemistry. D. variabilis* females were forcibly detached from their hosts 5–6 days postfeeding and inoculated with dopamine, reserpine, or Shen's solution. The tests were limited to these three treatments. Twenty-four hours postinoculation, the ticks were sacrificed, and the synganglion and body sections containing foveal glands and foveae dorsales were excised. Whole mounts and cryostat sections of these organs were prepared. The tissues were treated immediately for demonstration of monoamine histofluorescence using the sucrose–potassium phosphate–glyoxylic acid (SPG) method of De la Torre (1980). Slides were viewed with a Nikon Optiphot compound microscope using the epifluorescent attachment (violet filter, 470 nm filter) within 4 hr of staining. To calibrate the test, samples of mouse brain were stained and examined under epifluorescence, and the procedure was refined until the intensely bright reactive spots against a relatively dark background resembled that described by the author.

*Electron Microscopy. D. variabilis* females were inoculated with dopamine and reserpine in the same manner as ticks used in the histochemical analysis described above. Twenty-four hours postinoculation, ticks were prepared for ultrastructural study in accordance with standard electron microscopy techniques (Dawes, 1979). Postfixation (osmication), dehydration, and embedding were done in accordance with previously described techniques (Dawes, 1979). Sections were cut at 0.1 $\mu$m on an LKB-3 ultramicrotome (LKB Instruments,

Rockville, Maryland), mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi model HU-11B transmission electron microscope.

Elemental analysis for chlorine was also used as an indicator of 2,6-dichlorophenol, since the organically bound chlorine is not removed by the fixation, dehydration, or embedding techniques (Sonenshine et al., 1977). For elemental analysis, excised foveal glands from treated and untreated ticks were prepared in a similar manner, but sections were cut at 0.2 $\mu$m, and the unstained sections were mounted on uncoated copper grids and coated with a Technics Hummer V Sputter Coater. Other sections were excised, fixed, and dehydrated as described above; dried in a Denton DCP-1 critical point dryer (Denton Vacuum Systems, Cherry Hill, New Jersey); and coated with gold–palladium. The coated specimens were mounted on metal stubs and examined with a JOEL Co. JSMU-3 scanning electron microscope (SEM). Following verification of the identity of the foveal glands, elemental analysis for chlorine was done with an EDAX energy-dispersive analyzer, model 9100, attached to the SEM and Texas Instruments Silent ASR data terminal for intervals of 1000 sec. Elemental analysis was also done with a Hitachi HU-11B transmission electron microscope fitted with the same analyzer and data terminal.

## RESULTS

*Effects on Sex Attractant Activity.* Bioassay of the treated females revealed highly significant reductions in sex attractant activity in females treated with reserpine, pilocarpine, or $\alpha$-methyl tyrosine prior to feeding, but not with any other compound or with reserpine inoculated into partially fed females (Table 1). The attractiveness of the females inoculated unfed with reserpine was reduced to 44.2%, as compared to 90.1% or 93.1% in the controls treated with Shen's solution or 1% saline, or the solvent used to solubilize reserpine. This reduction was highly significant ( $t = 9.98$, 490 *df*, $P < 0.001$). Similar reductions were noted when reserpine was administered topically or when pilocarpine or MTME was injected prior to feeding.

The results of tests to determine the ability of treated females to attract males at a distance are summarized in Table 2. Most males (more than 70%) oriented to the treated or control females when they approached within 2 or 3 cm, except in the case of ticks treated with reserpine (intracoelomic injections) or MTME. In these cases, few males oriented at a distance of 3 cm; most males failed to orient until they came within 1 cm. However, the females treated by microinjection of reserpine under the scutum apparently were unaffected; evidently, the reserpine was not absorbed into the tick tissues in these individuals.

*Effects on Feeding.* Inoculation of 5-$\mu$l aliquots of Shen's solution had no significant effect on the engorged weight of the treated ticks (Table 3). Similarly,

TABLE 1. RESPONSES OF SEXUALLY ACTIVE MALE *Dermacentor variabilis* TO PARTIALLY FED FEMALES FOLLOWING INOCULATION OF PHARMACOLOGICALLY ACTIVE COMPOUNDS[a]

| Treatment administered | No. females | No. males | No. trials | Male response (%) | |
|---|---|---|---|---|---|
| | | | | Orientation | Attempted copulation |
| Reserpine | | | | | |
| Inoculated unfed | 6 | 40 | 120 | 44.2 | 42.5 |
| Topical[b] | 11 | 89 | 257 | 36.7 | 34.8 |
| Inoculated part fed | 11 | 78 | 228 | 85.1 | 74.2 |
| MTME | 6 | 30 | 93 | 32.2 | 32.2 |
| Dopamine | 10 | 99 | 296 | 95.3 | 88.7 |
| Cyclic AMP | 8 | 78 | 234 | 88.5 | 80.4 |
| Serotonin | 10 | 96 | 284 | 84.2 | 80.6 |
| 6-OH-Dopamine | 12 | 114 | 332 | 79.5 | 73.2 |
| Pilocarpine | 12 | 60 | 103 | 37.9 | 37.9 |
| Acetylcholine | 13 | 35 | 57 | 68.9 | 65.0 |
| Shen's[c] | 15 | 125 | 374 | 90.1 | 87.4 |
| Prop. glycol: | | | | | |
| Shen's control | 10 | 100 | 288 | 93.1 | 89.6 |
| DMSO control[d] | 6 | 30 | 72 | 87.3 | 85.7 |

[a]Inoculated as unfed females 24–48 hr prior to attachment to hosts. All inoculations were $10^{-3}$M in 5-$\mu$l volumes administered 24–48 hr prior to feeding, unless indicated otherwise; bioassays were done on the 7th day of feeding.
[b]$5 \times 10^{-2}$M reserpine dissolved in DMSO and applied topically to each female, 2 $\mu$l/tick; controls with DMSO only, 2 $\mu$l/tick.
[c]Includes some specimens inoculated with 1% saline.
[d]Solvents used for reserpine treatment; control done with solvents only.

inoculation of serotonin, cyclic AMP, or MTME had no significant effect on the weights of partially fed ticks, at least not at the doses used. In contrast, inoculation of reserpine at doses of $1 \times 10^{-3}$ M result in significantly lower engorge ment weights when compared with the untreated controls ($t$ = 5.84, 151 *df,* $P < 0.001$) or the Shen's controls ($t$ = 3.68, 139 *df,* $P < 0.001$). The effects of treatment with dopamine were the opposite of those observed with reserpine. Females inoculated with dopamine at a concentration of $1 \times 10^{-3}$ M exhibited a significant increase in the 7th day engorgement weight when compared with either untreated controls ($t$ = 3.01, 135 *df,* $P < 0.01$) or with the Shen's controls ($t$ = 3.36, 123 *df,* $P < 0.01$) (Table 3).

*Histochemistry.* The presence of monoamines in the synganglia and the foveal glands was demonstrated by the SPG reaction. When viewed with the microscope and epifluorescence attachment, intensely bright clusters were vis-

TABLE 2. ORIENTATION (%) OF SEXUALLY ACTIVE *Dermacentor variabilis* MALES TO TREATED FEMALES IN RELATION TO DISTANCE[a]

| Start distance (cm) | Type of treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | Shen's | Prop. glycol Shen's | Acetylcholine | Reserpine (intra-coel.)[b] | Reserpine (microinj.)[c] | Pilocarpine | $\alpha$-Methyl tyrosine |
| 3 | 62 | 45.0 | 47.1 | 10.8 | 33.3 | 53.4 | 10.0 |
| 2 | 34 | 45.0 | 30.0 | 27.7 | 36.7 | 30.0 | 16.7 |
| 1 | 2 | 6.7 | 12.9 | 27.7 | 16.7 | 8.3 | 26.7 |
| 0 | 2 | 3.3 | 10.0 | 33.8 | 13.3 | 8.3 | 46.7 |

[a] Each female was tested with five males; five females were used for each experiment. All inoculations were $10^{-3}$M in 5-$\mu$l volumes administered 24–48 hr prior to feeding. Bioassay were done on the 7th day of feeding.
[b] Intracoelomic inoculations.
[c] Inoculations administered to the scutal-capitular foramen with a glass micropipet and microinjector.

TABLE 3. EFFECT OF MONOAMINES AND OTHER PHARMACOLOGICALLY ACTIVE COMPOUNDS ON ENGORGEMENT WEIGHT OF VIRGIN *Dermacentor variabilis* FEMALES[a]

| Type of treatment | $N$ | Part-fed female weight (mg) (mean ± SD) |
|---|---|---|
| Untreated | 72 | 99.02 ± 36.32 |
| Shen's controls | 60 | 93.28 ± 44.42 |
| Cyclic AMP | 42 | 96.48 ± 33.34 |
| Serotonin | 46 | 82.56 ± 35.11 |
| Reserpine | 81 | 67.62 ± 35.70 |
| Dopamine | 65 | 118.04 ± 37.44 |
| Acetylcholine | 41 | 65.54 ± 32.34[b] |
| Pilocaarpine | 38 | 78.95 ± 45.63[b] |
| MTME | 43 | 98.58 ± 38.98 |

[a]All inoculations were $10^{-3}$M in 5-$\mu$l volumes administered 24–48 hr prior to feeding.
[b]Significantly different from Shen's controls, $P < 0.01$.

ible in the cortical zone of the synganglion (Figure 1) and in the ductular zone of the foveal glands (Figure 2). The contrasts between the intensely bright reactive sites and the nonspecific generalized histofluorescence were much more obvious to the observer when viewed with the microscope or photographed in color than when photographed in black and white.

The results of histochemical analysis of the synganglia and foveal glands from ticks treated with dopamine, reserpine, or Shen's solution are summarized in Table 4. The synganglia of ticks treated with reserpine had significantly fewer catecholamine-positive responses than those treated with dopamine or Shen's solution ($\chi^2 = 5.14$, 1 *df*, $P < 0.05$). Ticks treated with dopamine had virtually the same number of positive reactions as those treated with Shen's solution. Foveal gland samples treated with Shen's solution also revealed a high frequency of positive responses, although not as great as that observed with the synganglia. No detectable SPG-positive granules were observed in the foveal gland specimens following treatment with reserpine. Histofluorescence was observed only in intact specimens, i.e., foveal glands attached to the foveae dorsales.

*Effects on Pheromone Gland Ultrastructure and Chlorine Content.* When examined with the transmission electron microscope, secretory lobes of feeding females showed greatly reduced secretory droplet content following treatment with reserpine (Figure 3). Some of the lobes are virtually devoid of droplets. Numerous circular inclusion bodies (arrows), characteristic of intracellular autolytic processes, also appear (Figures 3 and 4). All lobes are not equally affected, although even those lobes with secretory droplets show lower counts than lobes of the glands from control specimens. The mean droplet count in specimens treated with reserpine was 11.87 droplets/100 $\mu m^2$ (range 2.5–50.6, $N$ =

FIG. 1. Photograph of the cortical zone in or near one of the pedal ganglia of a tick synganglion, illustrating catecholamine-related histofluorescence. The specimen was a partially fed female *D. variabilis* inoculated with Shen's physiological solution prior to feeding. The synganglion was excised, processed by the SPG technique (De la Torre, 1980), and photographed with an epifluorescent attachment (see text for details). The intensely bright areas are believed to represent catecholamine-reactive sites against a faint, nonspecific fluorescence. The contrast between the intensely bright reactive sites and the nonspecific histofluorescent background is not as great with black and white photography as it is when seen by the observer or recorded in color (1000×).

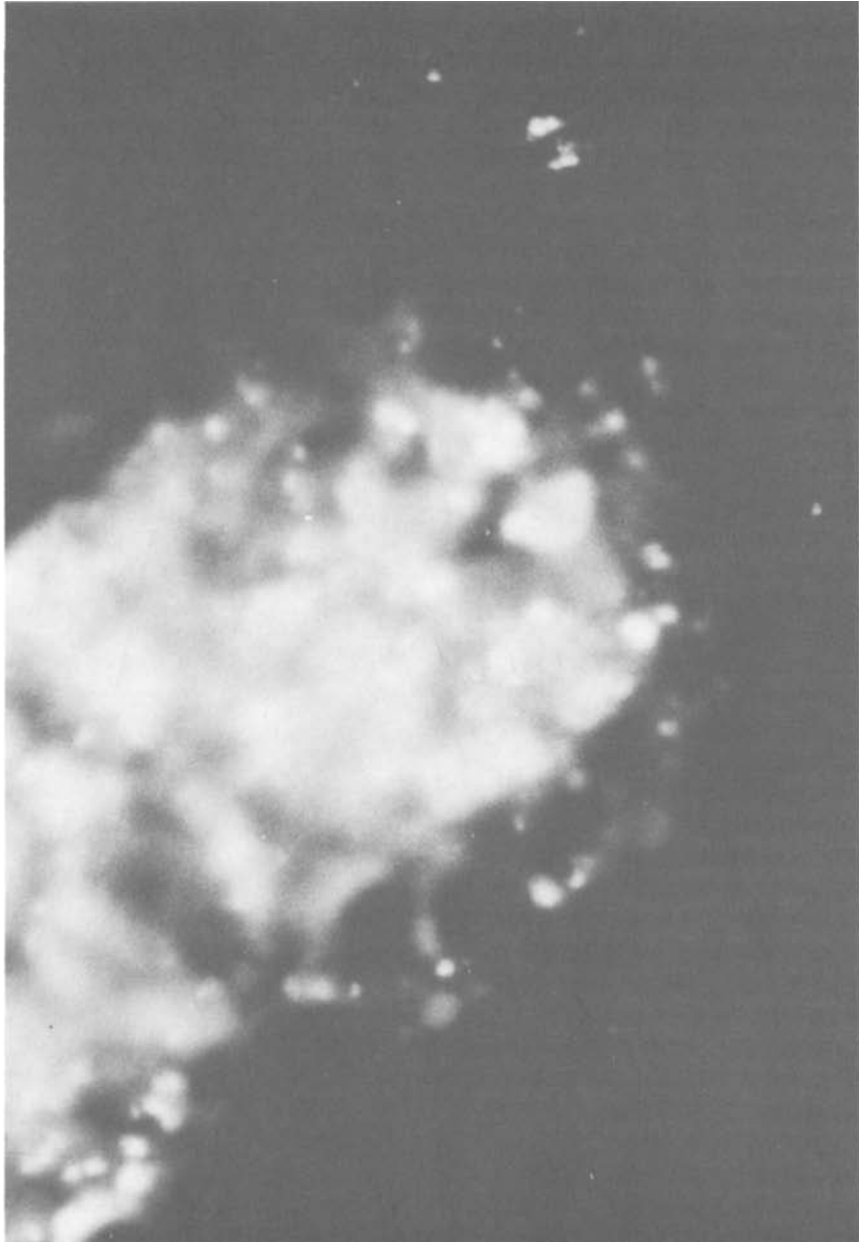

FIG. 2. Photograph of the foveal gland lobes and ductular zone attached to the underlying fovea of a partially fed female *D. variabilis* inoculated with $1 \times 10^{-3}$M dopamine prior to feeding. The specimens were processed and photographed as described above. The catecholamine reactive sites are believed to be the intensely bright clusters, partially obscured in some areas by a generalized histofluorescence (400×).

TABLE 4. SUMMARY OF HISTOCHEMICAL REACTIONS (SPG METHOD)[a] FOR MONOAMINES IN PARTIALLY FED *D. variabilis* FEMALES FOLLOWING TREATMENT WITH DOPAMINE OR RESERPINE[b]

| Treatment type | No. treated | No. reactions observed (%) | | |
|---|---|---|---|---|
| | | Strong | Weak | None |
| A. Synganglion | | | | |
| Reserpine | 14 | 4(28.6) | 3(21.4) | 7(50.0) |
| Dopamine | 13 | 10(76.9) | 1(7.7) | 2(15.4) |
| Shen's | 6 | 5(83.0) | 1(17) | 0(0.0) |
| B. Foveal Glands | | | | |
| Reserpine | 7 | 0(0.0) | 0(0.0) | 7(100) |
| Shen's | 10 | 5(43.0) | 2(20.0) | 3(30.0) |

[a] Sucrose-phosphate glyoxylic acid.
[b] All inoculations were $10^{-3}$M in 5-μl volumes administered 24–48 hr prior to feeding unless indicated otherwise.

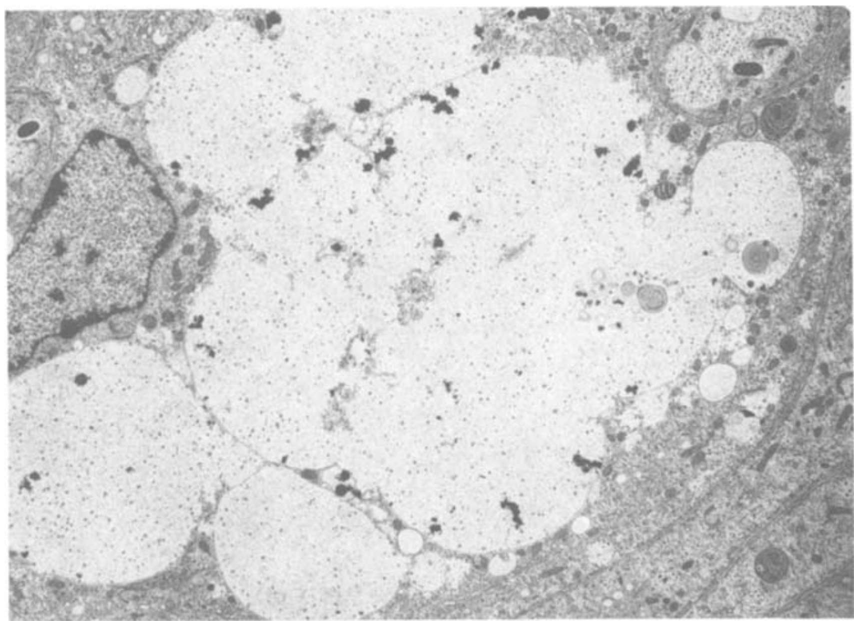

FIG. 3. Transmission electron micrograph of a lobe of the foveal gland from a *D. variabilis* female inoculated with a $1 \times 10^{-3}$M solution of reserpine prior to feeding. The lobe is almost entirely devoid of secretory droplets. Few lobes were found with abundant secretory droplets (4600×).

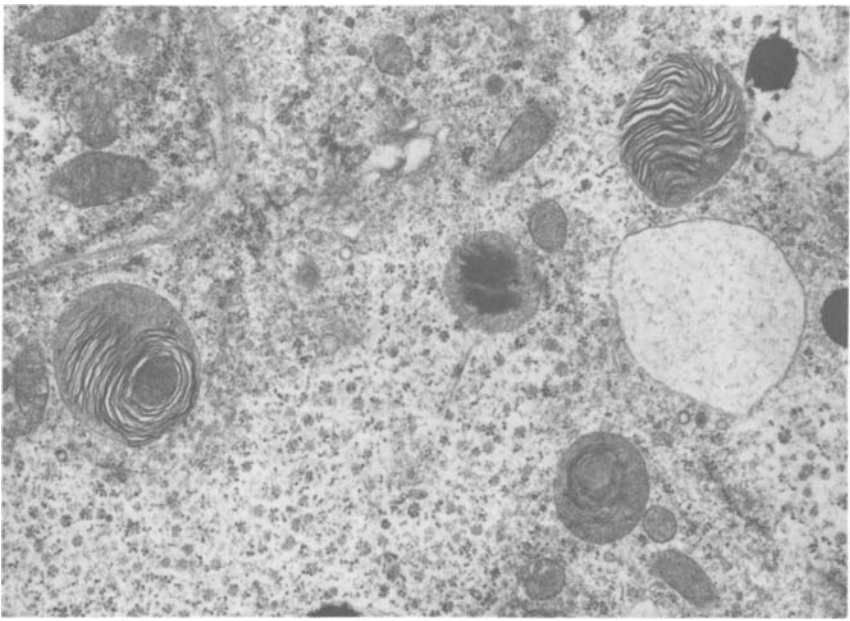

FIG. 4. Enlargement of a section through the lobe shown in Figure 3 illustrating the occurrence of circular bodies believed representative of autolytic processes, reflecting deterioration of the affected cell (20,800×).

5); the mean droplet count was 37.64 droplets/100 $\mu m^2$ (range 22.9–58.5, $N = 2$) in specimens treated with dopamine, and 46.74 droplets/$\mu m^2$ (range 24.3–69.18, $N = 2$) in specimens treated with Shen's solution. Little apparent difference in droplet content between either of the latter treatment types could be discerned. Figure 5 illustrates the appearance of the secretory lobes of the control (inoculated with Shen's solution); samples of the dopamine-treated specimens, which were indistinguishable from the controls, are not shown.

The results of elemental analysis for chlorine in the pheromone glands (secretory lobes only) of treated ticks are summarized in Table 5. The procedure for probing the tissues is illustrated in Figure 6. In the first set of samples, the values shown represent the total chlorine counts, expressed as percentages of the total osmium counts; each value is for a different specimen. Chlorine was significantly more abundant in the specimens treated with dopamine than in those treated with reserpine ($t = 4.98$, 8 *df*, $P < 0.01$). In the second set of samples, the values shown represent only the total chlorine counts. Chlorine was significantly reduced in the reserpine-treated specimens compared with the controls. Attempts to analyze samples treated with dopamine were unsuccessful.

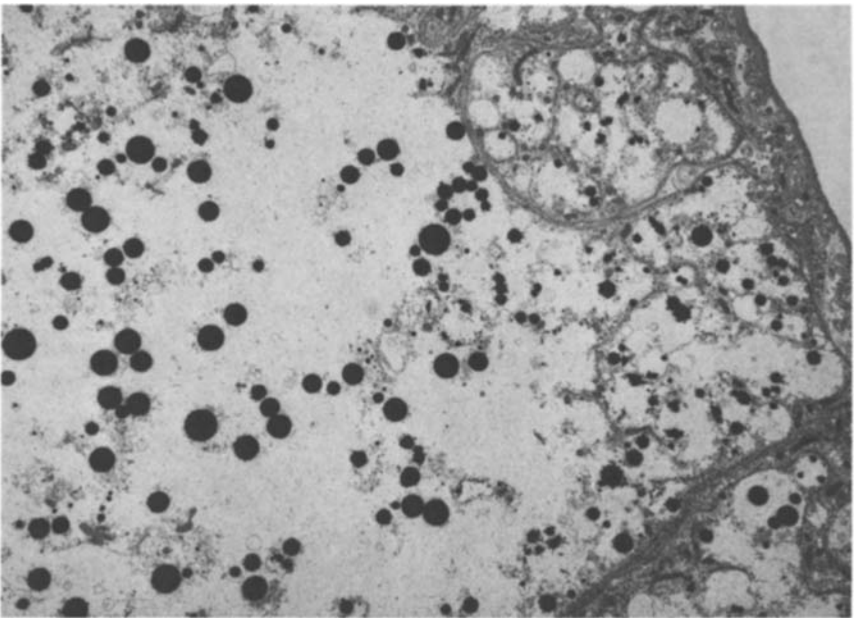

FIG. 5. Transmission electron micrograph of a section through a lobe of the secretory area of the foveal gland of a partially fed *D. variabilis* female inoculated with Shen's solution (control). The abundant occurrence of electron-dense osmiophilic secretory droplets is characteristic of these tissues in the feeding, pheromone-secreting females (6200×).

*Effect on Sex Pheromone Content.* The results of studies to determine the effects of different pharmacologically active compounds on tick sex pheromone content are summarized in Table 6. Part A presents results obtained with gas chromatography method 1; part B, with method 2. When analyzed by method 1, the 2,6-DCP (sex pheromone) content of untreated ticks was found to average 0.16 ng/tick. Ticks treated with Shen's solution had a similar content, averaging 0.20 ng/tick. Treatment with cyclic AMP, acetylcholine, or serotonin did not appear to affect 2,6-DCP content; the concentrations of pheromone observed following those treatments were 0.15 and 0.20 ng/tick, respectively. A single sample of ticks treated with reserpine had only 0.04 ng/tick. In contrast, two of three samples of dopamine-treated females exhibited greatly elevated concentrations, 5.5 and 7.0 times the Shen's controls.

The studies with dopamine and reserpine were repeated, using method 2 for extraction and analysis of 2,6-DCP content. Samples of females treated with $1 \times 10^{-3}$M reserpine or Serapsil prior to feeding exhibited slight reductions in their 2,6-DCP content when compared with the controls, namely, 2.30 and 2.60

TABLE 5. CHLORINE CONTENT OF FOVEAL GLAND SECRETORY LOBES (PHEROMONE GLANDS) OF PARTIALLY FED *Dermacentor variabilis* FEMALES TREATED WITH DOPAMINE OR RESERPINE PRIOR TO FEEDING[a]

Part A. Assayed in SEM mode

| Specimen No. | Chlorine (as % total osmium) in tick glands: Tick specimens treated with Dopamine | Reserpine | Shen's | Empty area of section |
|---|---|---|---|---|
| 1 | 24.83 | 8.63 | 13.56 | 6.41 |
| 2 | 21.43 | 10.44 | | |
| 3 | 25.81 | 7.08 | | |
| 4 | 13.42 | | | |
| 5 | 19.86 | | | |
| 6 | 19.82 | | | |
| 7 | 17.84 | | | |
| $\bar{X} \pm$ SD | 20.93 ± 3.91 | 8.72 ± 1.74 | | |

Part B. Assayed in TEM mode

| Specimen No. | Total counts in different designated areas: Tick specimens treated with Saline | Reserpine | Empty area of section |
|---|---|---|---|
| 1 | 1529 | 482 | 134 |
| 2 | 1977 | 398 | |
| 3 | 2006 | 571 | |
| 4 | | 257 | |
| $\bar{X} \pm$ SD | 1837 ± 221.4 | 427 ± 115.7 | — |

[a] All inoculations were $10^{-3}$M in 5-μl volumes administered 24–48 hr prior to feeding unless indicated otherwise.

ng/tick for the reserpine-treated specimens vs. 3.6 ng/tick for the Shen's controls (Table 6, part B). However, treatment of partially fed females while they were attached or use of a reduced concentration ($0.5 \times 10^{-3}$M) did not deplete the sex pheromone content; in three separate samples, the amounts observed exceeded that of the controls, i.e., 8.0, 3.70, and 4.50 ng/tick, respectively. Attempts to administer reserpine via the capitular-scutal foramen were unsuccessful. Mortality was 93.8%; the 2,6-DCP content of 13 survivors (16.0 ng/tick) is probably not representative and is not considered further.

Treatment with two other compounds also resulted in reduced 2,6-DCP content, namely, pilocarpine and MTME. The 2,6-DCP content of females

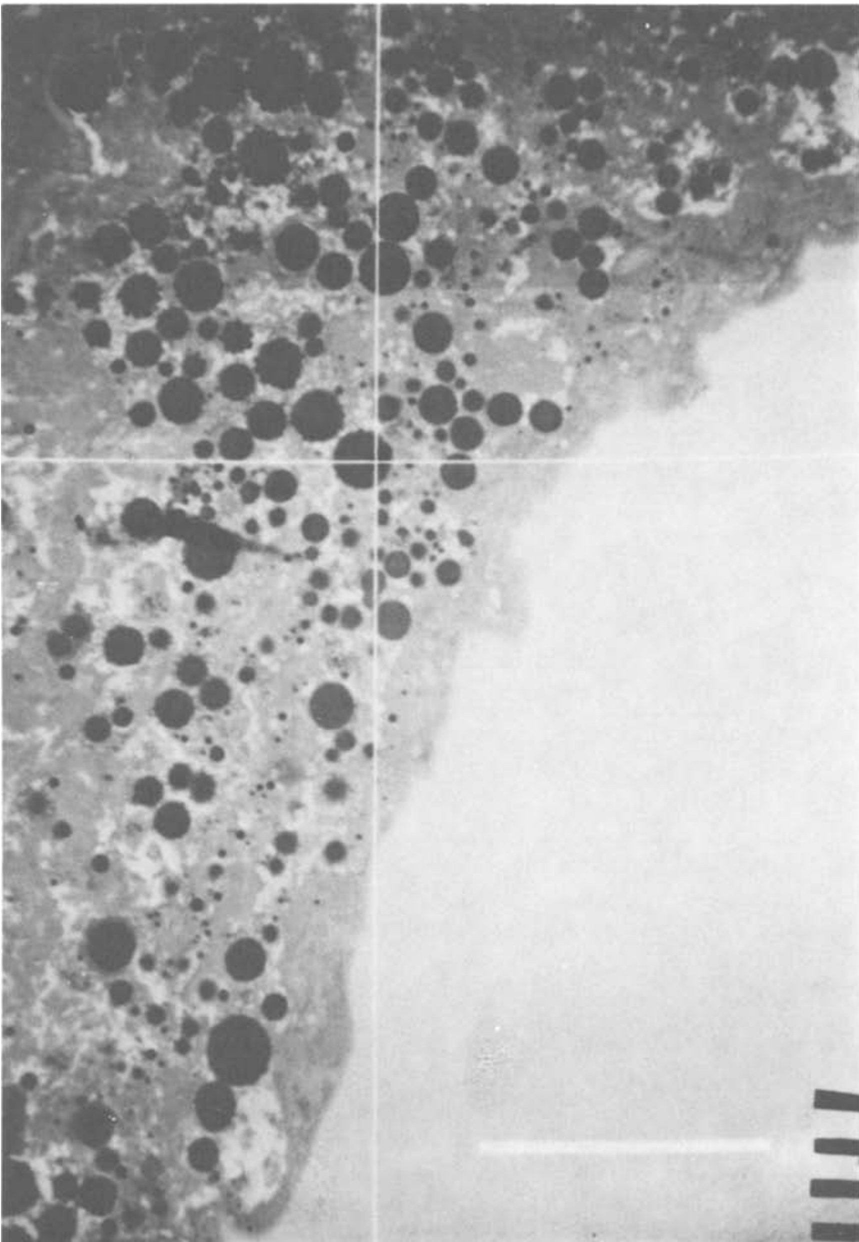

FIG. 6. Transmission electron micrograph of a section of a secretory lobe of the foveal gland of a partially fed *D. variabilis* female inoculated with Shen's solution prior to feeding. The intersection of the cross-hairs identifies one of the sites probed for quantification of chlorine content (2500×).

TABLE 6. EFFECTS OF MONOAMINES AND OTHER PHARMACOLOGICALLY ACTIVE COMPOUNDS ON SEX PHEROMONE (2,6-DICHLOROPHENOL) CONTENT IN *Dermacentor variabilis* FEMALES[a]

| Type of treatment | No. in sample | 2,6-DCP/tick (ng) |
|---|---|---|
| Part A (method 1) | | |
| Untreated | 225 | 0.16 |
| Shen's sol. | 176 | 0.20 |
| Cyclic AMP | 210 | 0.15 |
| Serotonin | 195 | 0.20 |
| Reserpine | 223 | 0.04 |
| Dopamine | 212 | 0.22 |
| Dopamine | 102 | 1.10 |
| Dopamine | 309 | 1.40 |
| Part B (method 2) | | |
| Untreated | 123 | 3.60 |
| Shen's sol. | 136 | 3.80 |
| Dopamine | 124 | 1.20 |
| Dopamine | 86 | 11.70 |
| Acetylcholine | 153 | 3.30 |
| Pilocarpine | 193 | 1.40 |
| Reserpine, $0.5 \times 10^{-3}$M | 27 | 4.50 |
| Reserpine | 97 | 2.30 |
| Reserpine[b] | 73 (part-fed) | 8.00 |
| Reserpine[c] | 93 (part-fed) | 3.70 |
| Serapsil | 57 | 2.60 |
| MTME | 81 | 0.90 |

[a] All females were unfed and approximately 2 weeks postemergence at the time of treatment. All inoculations were $10^{-3}$M in 5-$\mu$l volumes administered 24–48 hr prior to feeding unless indicated otherwise. All females were allowed to feed for 7 days before they were detached and placed in cold hexane.
[b] Injected while part fed, on day 6 of feeding, and removed 24 hr later.
[c] Injected while part fed, on day 5 of feeding, and removed 48 hr later.

treated with MTME was the lowest of all of the samples assayed, 0.9 ng/female (Table 6, part B).

Replication of the dopamine treatments gave inconsistent results. The 2,6-DCP content in one sample, 11.7 ng/female, was much greater than that of the Shen's or untreated controls, while the 2,6-DCP content of another sample, 1.20 ng/female, was much lower than the controls.

## DISCUSSION

These studies suggest a catecholamine-mediated excitation of sex pheromone activity in *D. variabilis*. Treatment of females with the catecholamine-

depleting agent reserpine led to a highly significant reduction in sex attractant activity when this compound was administered to unfed females, but not to partially fed ticks. Attempts to enhance this effect by increasing the dose led to 100% tick mortality. Treatment of partially fed females was ineffective, probably due to excessive dilution. Reductions in foveal gland chlorine content, secretory droplet content, and histofluorescence following reserpine treatment also indicated catecholamine mediation of this activity. The 2,6-DCP content of the reserpine-treated ticks was not affected as greatly, although the concentrations were less than that of the controls in most cases.

Much greater reductions in 2,6-DCP content were found following treatment with MTME. In contrast to reserpine, which inhibits catecholamine resorption, MTME antagonizes the synthesis of dopa from tyrosine by inhibiting tyrosine hydroxylase activity (Moore and Dominic, 1971). MTME is very soluble in aqueous media and may be expected to disperse readily throughout the body, facilitating its entry into axonal termini. In contrast, reserpine must be administered in oils or special formulations (e.g., Serapsil), and its distribution in vivo may not be uniform. In summary, treatment with two different catecholamine-inhibiting compounds provides evidence of reduction of sex pheromone activity, sex pheromone content, or both, findings consistent with the hypothesis of catecholaminergic mediation of pheromone gland activity. The reasons for the effects of pilocarpine are unclear and should be investigated further.

The foveal nerves, which innervate the tick pheromone glands, contain axons rich in neurosecretory and/or neurosecretomotor substances. In *D. variabilis* (Sonenshine et al., 1981) and *Hyalomma dromedarii* (Sonenshine et al., 1983), these nerves are found in the ductular zone. In both species, electron opaque and electron lucent vesicles occur, characteristic of neuropeptide and catecholamine transport vesicles, respectively. Further study of their figures indicates that the dense core vesicles are ca. 500 Ångstroms, the size typical of catecholamine-containing vesicles, in contrast to the neurosecretory vesicles, ca. 1000 Ångstroms. The histofluorescence studies reported in this paper are consistent with the evidence implicating catecholamine vesicles in the ductular zone of these glands; histofluorescence, indicating monoamine presence, was found almost entirely in the specimens where the glands remained attached to the cuticle. The histofluorescent reaction in the vicinity of the pedal ganglia of the synganglion (Figure 1) resembles that described by Binnington and Stone (1977) for *Boophilus microplus*.

Catecholamine stimulation of tick salivary gland secretion is well known and has been the subject of intensive study. Physiological evidence supports a neural stimulatory mechanism rather than a hormonal mechanism for initiating glandular activity. Copious secretion is elicited by dopamine, epinephrine, and norepinephrine (Kaufman and Sauer, 1982). Thus, neural regulation of glandular activity, mediated by catecholamines, is well known in ticks and may be inferred for other tick glandular systems as well.

The occurrence of catecholamines in the foveal glands supports the hypothesis of adrenergic regulation of sex pheromone activity. Treatment of ticks with reserpine led to reduced chlorine content in the secretory droplets and extensive depletion of these lipid formations, but did not consistently reduce the 2,6-DCP content. However, 2,6-DCP content was reduced in all three samples treated with $1 \times 10^{-3}$M reserpine, pilocarpine, or MTME and was elevated greatly in three of the five samples treated with dopamine. In contrast, treatment with other compounds had no apparent effect.

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# HPLC SEPARATION AND WAVELENGTH AREA RATIOS OF MORE THAN 50 PHENOLIC ACIDS AND FLAVONOIDS

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**Abstract**—Relative retention times and wavelength area ratios for over 50 standard compounds were calculated using reverse-phase HPLC. The standard compounds analyzed included benzoic acids, cinnamic acids, benzene carboxylic acids, acetic acids, coumarins, benzaldehydes and a variety of flavonoid compounds including flavanones, flavones, isoflavones, and their glycosides. Each standard compound was chromatographed by three different gradient elutions. Compounds were detected by UV absorption at 254 nm and 280 nm. Relative retention times with respect to two different internal references and the 254nm:280nm wavelength area ratio was determined for each standard. Soybean root and seed extracts were analyzed for the presence of the standard compounds using the chromatographic conditions described.

**Key Words**—HPLC, retention time, area ratio, phenolic acids, flavonoids, soybean, *Glycine max*.

## INTRODUCTION

HPLC has become a popular analytical tool for the separation of complex mixtures of metabolic intermediates. Phenolic acids have been implicated as active allelochemicals from living and decomposing plant tissues and are often precursors to flavonoids (Vickery and Vickery, 1981; Prikryl and Vancura, 1980; Bokari et al., 1979; Kuc, 1972; Rovira, 1969). Roston and Kissinger (1982) review recent work on the use of HPLC in the determination of phenolic acids, including those endogenous to plant material. They draw attention to the fact that many of the communications dealing with bonded-phase separation of mixtures of phenolic acids have also considerable flavonoid compounds because the



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two are often found in a common matrix. However, the greater hydrophobicity of the larger flavonoid molecules necessitates the use of gradients if they are to be determined simultaneously with phenolic acids. Several researchers have analyzed soybeans by HPLC for these types of compounds (Eldridge, 1982; Hardin and Stutte, 1980; Lookhart et al., 1978; Murphy and Stutte, 1978). In this study over 50 standard phenolic compounds, as well as soybean root and seed extracts, were analyzed by a modification of the HPLC procedure of Granato et al. (1983).

The procedures described in this paper attempt to circumvent the possibility that two or more compounds may have similar retention times (coelution) under a specific gradient analysis by measuring the absorbance of the sample simultaneously at two different wavelengths so that a wavelength area ratio could be determined for each component of the sample and by analyzing each sample by three different gradient conditions. To correct for any potential variations in column conditions, and to maximize the utility to other workers, retention times relative to internal references are reported rather than absolute retention times. Relative retention times are the most reliable index for compound identification (Johnson and Stevenson, 1978).

## METHODS AND MATERIALS

*Apparatus.* The liquid chromatograph system employed a Beckman model 420 controller/programmer equipped with Beckman models 100A and 110A solvent metering systems, a Beckman 25-cm $C_{18}$ Ultrasphere ODS column, and a Micromeritics 725 autoinjector with a 20-$\mu$l sample loop. Detection was by means of two Beckman variable wavelength detectors (models 100-40 and 100-10). Retention times and peak areas were obtained with two recording integrators (Beckman model C-R1A and Hewlett Packard 3390A). The two detectors were connected in series. Detection was achieved first at 254 nm by the model 100-40 detector and recorded by the Beckman C-R1A integrator. Approximately 0.1 min later detection was achieved at 280 nm by the model 100-10 detector and recorded by the HP 3390A integrator. The range for both detectors was 0.05, the absorbance mode was 0–2, and the time constant was 0. During the analyses, the pressure varied from approximately 13,700 $kP_a$ (at 10% B and a flow rate of 1.00 ml/min) to approximately 26,110 $kP_a$ (at 100% B and a flow rate of 1.20 ml/min).

*Chemicals.* Table 1 lists the source of the chemical standards and solvents. All chemicals were purchased as high-purity standards and were used without further purification. Solvents were HPLC spectral grade, and distilled water was deionized before use. All solvent ratios are given on a volume basis.

*Plant Material.* Wells II soybean [*Glycine max* (L.) Merrill] roots were taken from greenhouse-grown plants in the R-2 stage of growth (Fehr and Caviness, 1977) and analyzed. Root tissue was extracted by dimethyl sulfoxide

TABLE 1. STANDARD COMPOUNDS AND SOLVENTS

| Common name | Chemical name | Abbreviation | Source[a] |
|---|---|---|---|
| STANDARD COMPOUNDS | | | |
| Benzoic acids | | | |
| Gallic acid | 3,4,5-trihydroxybenzoic acid | 3,4,5-THBA | S |
| α-Resorcylic acid | 3,5-dihydroxybenzoic acid | 3,5-DHBA | S |
| Protocatechuic acid | 3,4-dihydroxybenzoic acid | 3,4-DHBA | S |
| Gentisic acid | 2,5-dihydroxybenzoic acid | 2,5-DHBA | S |
| β-Resorcylic acid | 2,4-dihydroxybenzoic acid | 2,4-DHBA | S |
| | 4-hydroxybenzoic acid | 4-HBA | A |
| Salicylic acid | 2-hydroxybenzoic acid | 2-HBA | A |
| | 4-ethoxybenzoic acid | 4-EBA | E |
| | 4-aminobenzoic acid | 4-ABA | A |
| Vanillic acid | 4-hydroxy-3-methoxybenzoic acid | 4-H,3-MBA | A |
| Syringic acid | 4-hydroxy-3,5-dimethoxybenzoic acid | 4-H,3,5-DMBA | A |
| Benzoic acid | | BA | A |
| Benzaldehydes | | | |
| | 4-hydroxybenzaldehyde | 4-HBald | S |
| | 2,3-dihydroxybenzaldehyde | 2,3-DHBald | S |
| Vanillin | 4-hydroxy-3-methoxybenzaldehyde | 4-H,3-MBald | S |
| Ethylvanillin | 4-hydroxy-3-ethoxybenzaldehyde | 4-H,3-EBald | E |
| Syringaldehyde | 4-hydroxy-3,5-dimethoxybenzaldehyde | 4-H,3,5-DMBald | M |
| Phenols | | | |
| Pyrogallol | 1,2,3-benzenetriol | pyrogallol | A |
| Catechol | 1,2-benzenediol | catechol | E |
| Benzene carboxylic acids | | | |
| Terephthalic acid | 4-benzene carboxylic acid | terephthalic | E |
| Phthalic acid | 1,2-benzene dicarboxylic acid | phthalic | E |
| Diphenic acid | 1,1′-biphenol-2,2′-dicarboxylic acid | diphenic | E |
| Pyromellitic acid | 1,2,4,5-benzene tetracarboxylic acid | pyromellitic | E |
| Acetic acid | | | |
| Homophthalic acid | 2-carboxyphenyl acetic acid | homophthalic | K |
| | 3-indole acetic acid | IAA | E |
| | 1-naphthalene acetic acid | NAA | E |
| Cinnamic acids | | | |
| Caffeic acid | 3,4-dihydroxycinnamic acid | 3,4-DHCA | A |
| Ferulic acid | 4-hydroxy-3-methoxycinnamic acid | 4-H,3-MCA | A |
| *p*-Coumaric acid | 4-hydroxycinnamic acid | 4-HCA | A |
| *m*-Coumaric acid | 3-hydroxycinnamic acid | 3-HCA | S |
| *o*-Coumaric acid | 2-hydroxycinnamic acid | 2-HCA | M |
| Cinnamic acid | 3-phenyl-2-propenoic acid | CA | A |
| Coumarins | | | |
| Scopoletin | 7-hydroxy-6-methoxycoumarin | 7-H,6-MC | S |
| Umbelliferone | 7-hydroxycoumarin | 7-HC | S |
| | 4-hydroxycoumarin | 4-HC | S |
| Coumarin | 1,2-benzopyrone | coumarin | A |

TABLE 1. Continued

| Common name | Chemical name | Abbreviation | Source[a] |
|---|---|---|---|
| STANDARD COMPOUNDS Cont. | | | |
| Flavanones | | | |
| Naringenin | 4′,5,7-trihydroxyflavanone | naringenin | S |
| Hesperetin | 4′-methoxy-3′,5,7-trihydroxyflavanone | hesperetin | S |
| *d*-Catechin | 3,3′,4′,5,7-pentahydroxyflavanone | d-catechin | S |
| Flavanone glycosides | | | |
| Naringin | naringenin-7-rhamoglucoside | naringin | A |
| Hesperedin | hesperetin-7-rutinoside | hesperedin | S |
| Flavones | | | |
| Kaempferol | 3,4′,5,7-tetrahydroxyflavone | kaempferol | S |
| Morin | 2′,3,4′,5,7-pentahydroxyflavone | morin | S |
| Quercetin | 3,3′,4′5,7-pentahydroxyflavone | quercetin | S |
| Myricetin | 3,3′,4′,5,5′,7-hexahydroxyflavone | myricetin | S |
| Flavone glycosides | | | |
| Rutin | quercetin-3-rutinoside | rutin | S |
| Isoflavones | | | |
| Daidzein | 4′,7-dihydroxyisoflavone | daidzein | I |
| Genistein | 4′,5,7-trihydroxyisoflavone | genistein | K |
| Coumestans | | | |
| Coumestrol | dihydrocoumarinocoumarone | coumestrol | P |
| Other standards | | | |
| | methyl-4-aminobenzoate | M-4-ABenz | E |
| Indole | 1-benzo [b] pyrrole | indole | A |
| Phloretin | dihydronaringenin | phloretin | S |
| Uracil | 2,4-pyrimidinedione | uracil | N |
| P-Anisidine | 4-methoxyaniline | anisidine | E |
| Shikimic acid | trihydroxycyclohexenecarboxylic acid | shikimic | S |
| Chlorogenic | 3-caffeoylquinic acid | chlorogenic | A |
| SOLVENTS | | | |
| Glacial acetic acid | | | F |
| Ammonium acetate | | | F |
| *n*-Butanol | | | B |
| Methanol | | | B |
| Dimethyl sulfoxide | | DMSO | F |
| Water | | | W |

[a] A = Aldrich Chemical Company; B = Burdick & Jackson Laboratories Inc.; E = Eastman Kodak Company; F = Fisher Scientific Company; I = from Dr. John Ingham, University of Reading; K = K & K Company; M = Mann Research Laboratories; N = Nutritional Biochemicals Corporation; P = Pfaltz & Bauer, Inc.; S = Sigma Chemical Company; W = purified by a Millipore Milli-Q Water Purification System.

(DMSO) according to methods previously described by Granato et al. (1983). The seed extract was from the soybean cultivar Pella. Fifty seeds were cracked by running them four times through a Wiley mill grinder with no retaining screen in place. The cracked seeds were mixed with DMSO in a 2 : 1 (w/w) ratio, and this mixture was shaken for 30 min (at low speed on an Eberbach shaker). The DMSO extract was decanted off, centrifuged (by a Damon IEC B-20A centri fuge) at 294,000 m/sec$^2$ for 10 min and filtered through a 5-$\mu$m Millipore filter. Prior to analysis two internal references, 2,4-DHBA and indole, were added to the soybean extracts.

*Separation Procedure.* The mobile phase consisted of solvents A and B. Solvent A contained 98% water and 2% glacial acetic acid in 0.018 M ammonium acetate. Solvent B was 70% solvent A and 30% organic solvent. The organic solvent contained 82% methanol, 16% *n*-butanol, and 2% glacial acetic acid in 0.018 M ammonium acetate. Solvent A was pumped by the model 100A pump and solvent B by the model 110A pump. All the standards were analyzed by the three gradient elutions. The initial flow rate for all gradients was 1.00 ml/min. The three gradient elutions consisted of the following steps:

For gradient 1: (a) 0.0–1.0 min isocratic at 10% B; (b) 1.0–21.0 min linear gradient from 10 to 25% B; (c) 21.0–36.0 min linear gradient from 25 to 45% B; (d) 36.0–56.0 min linear gradient from 45 to 100% B; (e) 50.0–50.15 min flow increased to 1.20 ml/min; (f) 82.0–82.15 min linear gradient from 100 to 10% B; (g) 92.0–92.15 min flow decreased to 1.00 ml/min; (h) at 99.0 min sample loop rinsed and gradient repeated.

For gradient 2: (a) 0.0–1.0 min isocratic at 20% B; (b) 1.0–21.0 min linear gradient from 20 to 35% B; (c) 21.0–36.0 min linear gradient from 35 to 45% B; (d) 36.0–56.0 min linear gradient from 45 to 90% B; (e) 50.0–50.15 min flow increased to 1.20 ml/min; (f) 60.0–61.0 min linear gradient from 90 to 100% B; (g) 85.0–85.15 min linear gradient from 100 to 20% B; (h) 92.0–92.15 min flow decreased to 1.00 ml/min; (i) at 99.0 min sample loop rinsed and gradient repeated.

For gradient 3: (a) 0.0–1.0 min isocratic at 20% B; (b) 1.0–21.0 min linear gradient from 20 to 45% B; (c) 21.0–61.0 min linear gradient from 45 to 90% B; (d) 50.0–50.15 min flow increased to 1.20 ml/min; (e) 61.0–66.0 min linear gradient from 90 to 100% B; (f) 85.0–85.15 min linear gradient from 100 to 20% B; (g) 92.0–92.15 min flow decreased to 1.00 ml/min; (h) at 99.0 min sample loop rinsed and gradient repeated.

Each standard compound was first subjected to analysis by gradient 1 to determine its absolute retention time. Standard mixes were made which grouped together up to 10 standard compounds whose retention times differed sufficiently to allow for good resolution. These standard mixes were then subjected to analysis by the three gradients. The absolute retention times of some of the standard compounds analyzed both individually and in their respective standard mix were compared to verify that their absolute retention times did not change upon mixing of the standard compounds.

TABLE 2. RELATIVE RETENTION TIMES AND WAVELENGTH AREA RATIOS FOR STANDARD COMPOUNDS

| Standard Compound | Standard Mix[a] | RRT(2,4-DHBA) gradient | | | RRT(indole) gradient | | | Wavelength area ratio (254nm : 280nm) | Peak area at 280nm[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 1 | 2 | 3 | | |
| 1. Uracil | D | sf[c] | sf | sf | sf | sf | sf | sf | sf |
| 2. Pyromellitic | E | sf | sf | sf | sf | sf | sf | sf | sf |
| 3. Pyrogallol | F | 0.38 | 0.47 | 0.49 | 0.10 | 0.10 | 0.11 | 4.2 | 1 |
| 4. 3,4,5-THBA | A | 0.40 | 0.45 [d] | 0.46 | 0.11 | 0.10 [d] | 0.11 | 0.6 | 750 |
| 5. 2,5-DHBA | G | 0.59 | 0.65 | 0.65 | 0.16 | 0.14 | 0.15 | 1 | 45 |
| 6. 3,5-DHBA | D | 0.65 | 0.63 | 0.65 | 0.18 | 0.14 | 0.15 | 6.0 | 110 |
| 7. 3,4-DHBA | C | 0.72 | 0.75 | 0.74 [d] | 0.20 | 0.16 | 0.17 [d] | 2.0 | 530 |
| 8. 4-ABA | B | 0.74 | 0.79 [d] | 0.79 [d] | 0.20 | 0.17 [d] | 0.19 [d] | 0.4 | 1900 |
| 9. Phthalic | E | 0.77 [d] | 0.84 [d] | 0.92 [d] | 0.21 [d] | 0.18 [d] | 0.21 [d] | 1.2 | 120 |
| 10. Catechol | G | 0.82 | 0.98 | 0.94 | 0.22 | 0.21 | 0.22 | 0.1 | 420 |
| 11. Anisidine | A | 1.00 | 1.00 | 0.95 | 0.27 | 0.21 | 0.22 | 0.1 | 190 |
| 12. 2,4-DHBA | B | 1.00 | 1.00 | 1.00 | 0.27 | 0.21 | 0.23 | 3.5 | 410 |
| 13. 4-HBA | C | 1.18 | 1.21 | 1.20 | 0.32 | 0.26 | 0.28 | 3.7 | 570 |
| 14. Terephthalic | F | 1.36 | 1.32 | 1.31 | 0.37 | 0.28 | 0.31 | 6.7 | 220 |
| 15. *d*-Catechin | D | 1.42 | 1.16 | 1.15 | 0.39 | 0.25 | 0.27 | 0.2 | 190 |
| 16. Shikimic | G | 1.47 | 1.49 | 1.44 | 0.40 | 0.32 | 0.34 | 0.1 | 18 |
| 17. 4-HBald | D | 1.47 | 1.49 | 1.44 | 0.40 | 0.32 | 0.34 | 0.3 | 2400 |
| 18. Chlorogenic | E | 1.48 | 1.28 | 1.27 | 0.41 | 0.27 | 0.30 | 1 | 300 |
| 19. 4-H,3-MBA | A | 1.55 | 1.52 | 1.46 | 0.43 | 0.32 | 0.34 | 2.0 | 590 |
| 20. 2,3-DHBald | B | 1.58 | 1.60 | 1.52 | 0.42 | 0.34 | 0.36 | 1.3 | 960 |
| 21. 3,4-DHCA | C | 1.76 | 1.67 | 1.63 | 0.48 | 0.34 | 0.37 | 0.6 | 890 |
| 22. 2-HBA | F | 1.80 | 1.95 | 1.89 | 0.49 | 0.41 | 0.44 | 0.2 | 260 |
| 23. 4-H,3,5-DHBA | A | 1.87 | 1.77 | 1.65 | 0.52 | 0.37 | 0.39 | 0.5 | 1000 |
| 24. Homophthalic | B | 1.92 | 1.89 | 1.79 | 0.52 | 0.40 | 0.42 | 1.0 | 110 |
| 25. 4-H,3,4-DHBald | B | 2.28 | 2.33 | 2.23 | 0.61 | 0.49 | 0.51 | 0.7 | 78 |
| 26. M-4-ABenz | A | 2.44 | 2.59 | 2.35 | 0.67 | 0.55 | 0.55 | 0.3 | 504 |
| 27. 7-HC | C | 2.49 | 2.58 | 2.35 | 0.67 | 0.55 | 0.55 | 0.5 | 500 |
| 28. 4-HCA | B | 2.53 | 2.66 | 2.44 | 0.68 | 0.56 | 0.58 | 0.2 | 2900 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 29. 4-H,3-MCA | A | 2.83 | 3.13 | 2.74 | 0.78 | 0.66 | 0.64 | 0.6 | 930 |
| 30. BA | F | 2.83 | 3.33 | 3.06 | 0.78 | 0.71 | 0.72 | 0.9 | 130 |
| 31. 7-H,6-MC | C | 2.86 | 3.08 | 2.68 | 0.78 | 0.65 | 0.63 | 1.2 | 380 |
| 32. 4-H,3MBald | G | 2.88 | 3.24 | 2.86 | 0.79 | 0.69 | 0.67 | 1.7 | 580 |
| 33. 3-E,4-HBald | D | 2.92 | 3.25 | 2.85 | 0.80 | 0.69 | 0.67 | 0.3 | 1300 |
| 34. 3-HCA | E | 3.02 | 3.44 | 3.08 | 0.83 | 0.73 | 0.72 | 0.4 | 2100 |
| 35. IAA | B | 3.06 | 3.44 | 3.03 | 0.82 | 0.73 | 0.72 | 0.5 | 600 |
| 36. Kaempferol | G | ur[e] | ur | ur | ur | ur | ur | ur | ur |
| 37. Coumarin | C | 3.43 | 4.14 | 3.60 | 0.93 | 0.88 | 0.84 | 0.4 | 1500 |
| 38. Myricetin | F | ur | ur | ur | ur | ur | ur | ur | ur |
| 39. 2-HCA | A | 3.57 | 4.57 | 3.93 | 0.98 | 0.97 | 0.92 | 0.4 | 1900 |
| 40. Indole | A | 3.66 | 4.73 | 4.27 | 1.00 | 1.00 | 1.00 | 0.6 | 410 |
| 41. Naringin | B | 3.80 | 4.99 | 4.03 | 1.02 | 1.06 | 0.95 | 0.2 | 450 |
| 42. Rutin | D | 3.80 | 5.09 | 4.27 | 1.04 | 1.08 | 1.00 | 2.3 | 190 |
| 43. 4-HC | E | 3.82 | 5.04 | 4.40 | 1.05 | 1.06 | 1.02 | 0.4 | 1300 |
| 44. Hesperidin | C | 3.84 | 5.13 | 4.29 | 1.04 | 1.09 | 1.00 | 0.2 | 400 |
| 45. Diphenic | B | 3.40 | 5.28 | 4.49 | 1.08 | 1.12 | 1.06 | 1.7 | 150 |
| 46. Daidzein | C | 4.41 | 5.99 | 5.89 | 1.20 | 1.26 | 1.37 | 2.4 | 460 |
| 47. CA | H | 4.46 | 6.00 | 5.85 | 1.21 | 1.27 | 1.36 | 0.4 | 2400 |
| 48. 4-EBA | A | 4.62 | 6.30 | 6.12 | 1.27 | 1.33 | 1.44 | 2.8 | 490 |
| 49. Morin | G | ur | ur | ur | ur | ur | ur | ur | ur |
| 50. Naringenin | B | 4.79 | 6.57 | 6.47 | 1.29 | 1.39 | 1.53 | 0.1 | 620 |
| 51. Hesperetin | C | 4.94 | 6.98 [d] | 7.09 | 1.34 | 1.74 [d] | 1.67 | 0.1 | 730 |
| 52. Quercetin | D | 4.95 | 7.10 | 7.21 | 1.35 | 1.51 | 1.69 | 2 | 2000 |
| 53. Genistein | A | 4.96 | 7.00 | 7.09 | 1.36 | 1.48 | 1.66 | 2.5 | 690 |
| 54. NAA | B | 5.09 | 7.04 | 6.95 | 1.37 | 1.49 | 1.64 | 0.4 | 520 |
| 55. Phloretin | C | 5.18 | 7.27 | 7.61 | 1.40 | 1.54 | 1.78 | 0.2 | 920 |
| 56. Coumestrol | E | 5.80 | 8.21 | 8.55 | 1.59 | 1.73 | 1.99 | 4.1 | 250 |

[a] Each standard mix was comprised of up to 10 standard compounds designated by the same letter.
[b] Peak area is in integration units $\times 10^{-3}$ for standard mixes containing 10 $\mu$g/ml of individual compounds.
[c] sf= No data reported because of solvent front interference.
[d] More than one peak was observed for this standard compound. RRT of major peak observed is recorded.
[e] ur = No data reported because of broad, undefined response.

The standards were dissolved in dimethyl sulfoxide and the concentration of each standard compound upon analysis was 10.0 μg/ml. Each standard mix also contained two internal references: 2,4-DHBA and indole. Both internal references absorbed well at the wavelengths of interest. 2,4-DHBA eluted early in the HPLC analyses, whereas indole eluted much later. The concentration of the two internal references upon analysis was 10.0 μg/ml and 5.0 μg/ml for 2,4-DHBA and indole, respectively.

*Calculations.* Peak retention times and peak areas were monitored and computed by integrating recorders. The relative retention time (RRT) for each standard compound with respect to each of the two internal references was calculated by dividing the absolute retention time (RT) of the standard compound by the RT of the internal reference. Table 2 lists the relative retention times of all the standard compounds. They are listed according to increasing RRT with respect to 2,4-DHBA for gradient 1. In instances where the standard compounds eluted at the solvent front, and those where the recorder failed to detect the standard compound because the peak was too broad, no RRT was calculated.

Since each sample was analyzed simultaneously at 254 nm and 280 nm, a ratio of the integrated area at 254 nm compared to 280 nm was calculated and is called the wavelength area ratio. For two or more detectors operated under fixed conditions the ratio of response of a single compound to two different wavelengths is characteristic of that compound (Johnson and Stevenson, 1978). Additional evidence for compound identification is possible when wavelength area ratios of a standard compound are very similar to those for an unknown of similar retention time. These ratios can best be obtained by connecting two detectors in series or by information obtained from a rapid scan detector that records absorbance and peak areas for two or more wavelengths simultaneously.

Because our studies used two absorbance detectors connected in series, each with a separate integrating device, a correlation between the peak areas (integration units) obtained from the two recording integrators was determined. The conversion factor for normalizing areas from recorder 1 to recorder 2 was 7.69. Thus the integrated areas of the peaks analyzed at 254 nm by the first recording integrator were adjusted by a factor of 7.69 to give normalized areas that correspond to the integrated areas had the analysis been done at 254 nm by the second recording integrator.

The normalized integrated area of each standard compound at the two wavelengths analyzed for each of the three gradients was determined. Thus three wavelength area ratios could be calculated for each standard compound. Table 2 lists the mean wavelength area ratio and the mean normalized integrated area (integration units) at 280 nm for each of the standard compounds.

## RESULTS AND DISCUSSION

Figure 1 shows a representative chromatogram of a standard mix detected at 280 nm. Table 2 reports retention times relative to two internal references

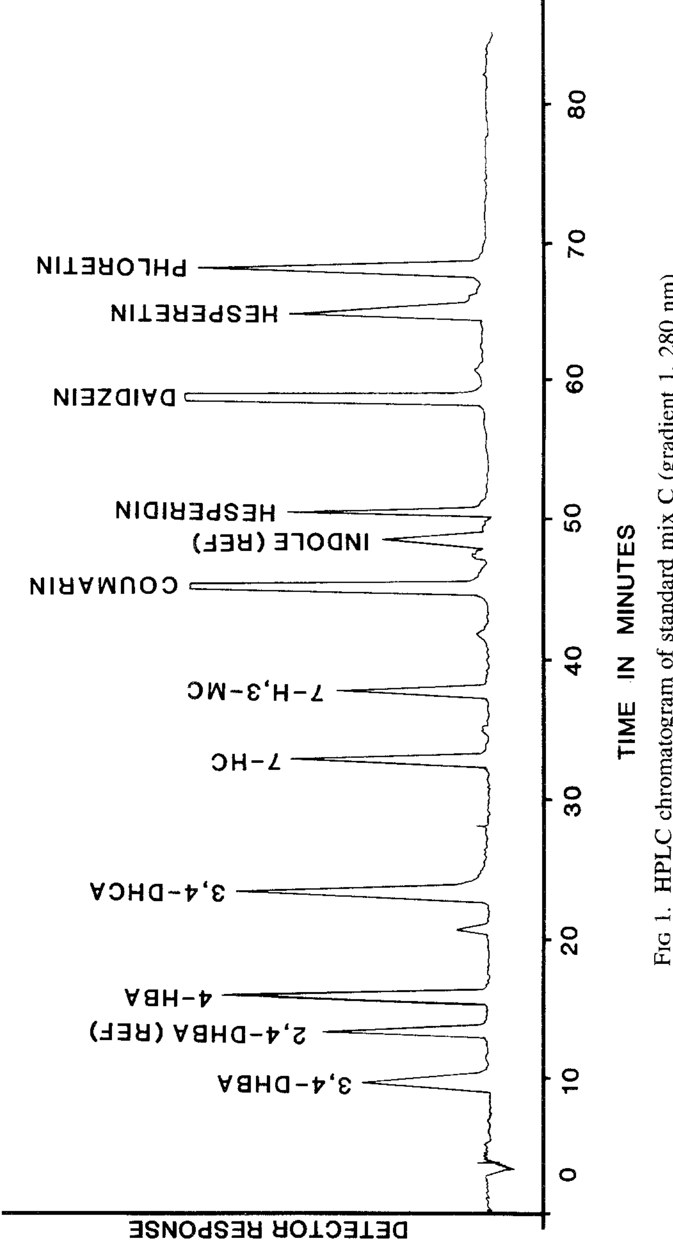


FIG 1. HPLC chromatogram of standard mix C (gradient 1, 280 nm).

and the 254nm:280nm wavelength area ratios for each of 56 standard compounds. The use of three gradients is intended to increase the probability that compounds will be properly identified. The data in Table 2 show that a single gradient of the type proposed would not allow separation of all compounds. Anisidine, for example, could not be distinguished from 2,4-DHBA on gradients 1 and 2 (RRT = 1.00), but it has a somewhat smaller RRT (0.95) on gradient 3. Because of the large number of compounds of interest in many characterization studies and in an effort to improve the probability of proper compound identification, the 254nm:280nm wavelength area ratios were also reported. Anisidine, for example, has a wavelength area ratio of 0.1 whereas 2,4-DHBA has a wavelength area ratio of 3.5. Comparison of wavelength area ratios in this case is a valuable tool in helping to establish compound identification.

There are at least two limitations to wavelength area ratios that should be noted. First, chromatograms from five of the 56 compounds tested resulted in two poorly resolved peaks from one or more of the gradients used. This may be the result of impure standards or such possibilities as isomers of the same standard that absorb differently at one wavelength than they do at another. In cases where two peaks were observed, the total peak area was used for the wavelength area ratioing (Table 2). When the two poorly resolved peaks occur, the wavelength area ratios are undoubtably less precise. Secondly, if the absorbance of an unknown is very small, the integrated peak area will be more subject to integration parameters and errors. The areas reported in Table 2 indicate the peak areas for some compounds are several orders of magnitude larger than the peak areas of compounds like pyrogallol, for example. Thus wavelength area ratios other than 254nm:280nm may be more appropriate for specific compounds. These techniques (multiple gradients and wavelength area ratios) are not intended to replace the value of additional compound identification methods such as mass spectrometry or infrared analysis. They can, however, serve to add evidence for positive identification and, in many cases, can be used to rule out tentative identifications made on a single chromatogram with a single detector.

Results shown in Table 2 indicate that the gradients used in this study will not detect 10 $\mu$g/ml uracil, pyromellitic acid, kaempferol, myricetin, and morin. If these compounds are of specific interest, modifications of the proposed gradients would be needed.

Figures 2 and 3 show examples of chromatograms obtained by applying the methods described to soybean root and seed extracts. Using the information in Table 2, we were able to determine that daidzein and genistein were present in soybean roots extracted with DMSO, and these same compounds were present in extracts from soybean seeds. Benzoic and cinnamic acids were not found in the free form in roots or in the seeds of soybeans.

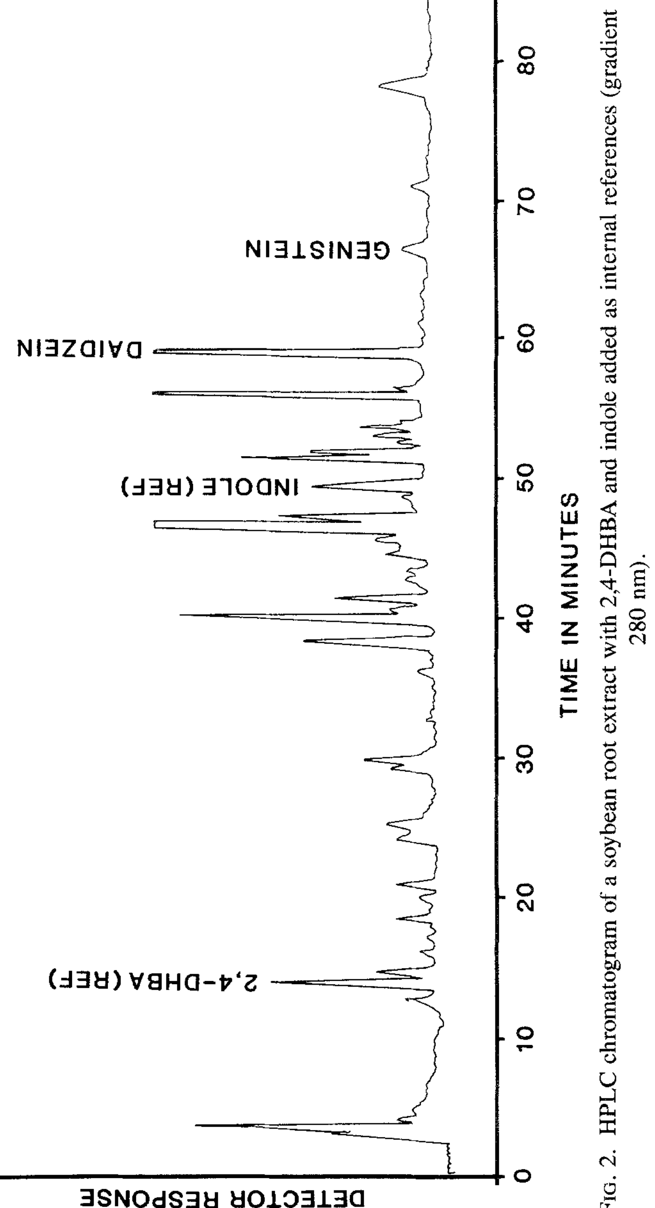


FIG. 2. HPLC chromatogram of a soybean root extract with 2,4-DHBA and indole added as internal references (gradient 1, 280 nm).

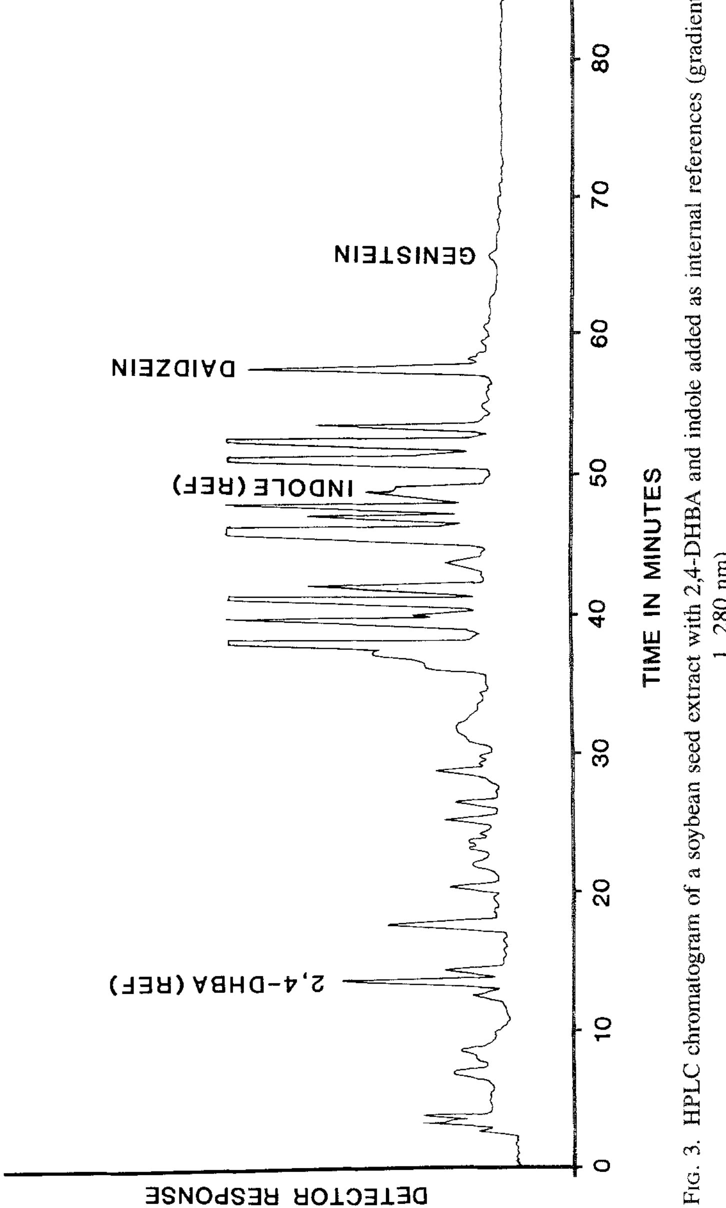


FIG. 3. HPLC chromatogram of a soybean seed extract with 2,4-DHBA and indole added as internal references (gradient 1, 280 nm).

The absence of benzoic, cinnamic, and related acids in the free form in soybean root and seed extracts is somewhat surprising because of the emphasis placed on these compounds as potential allelochemicals. Further isolation and evidence for positive identification of daidzein and genistein will be presented in a subsequent report. The methods reported in this paper should be applicable to extracts of a wide variety of biological materials. They allow detection of a large number of phenolic acids and flavonoids. Relative retention times with three different gradients and use of wavelength area ratios greatly improve the confidence of the analytical technique.

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# MALE OLIVE FRUIT FLY ATTRACTION TO SYNTHETIC SEX PHEROMONE COMPONENTS IN LABORATORY AND FIELD TESTS

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**Abstract**—Male olive fruit fly attraction to the four synthetic components of the female sex attractant pheromone was studied under laboratory and field conditions. In laboratory tests males responded to all four components tested separately. Component I, (1,7-dioxaspiro[5.5]undecane) was more attractive than any of the remaining three components alone, but a combination of all four was more attractive than component I alone. In field tests with polyethylene vials as pheromone dispensers, the complete mixture, although not statistically significant, was constantly more attractive to males than component I alone. A tendency of enhancement of attraction of component I by combining it with component II ($\alpha$-pinene) or III (*n*-nonanal) was also observed. In field tests with rubber septa as pheromone dispensers only component I was attractive. Mixtures containing component I were also attractive but not more attractive than component I alone. Evaporation rate and ratio of components as they come out of the dispenser appear to be critical for male response.

**Key Words**—*Dacus oleae,* olive fruit fly, Diptera, Tephritidae, pheromones, sex attractants, multicomponent pheromones, field tests of pheromones.

## INTRODUCTION

The sex attractant pheromone produced by female olive fruit flies, *Dacus oleae* Gmelin, was found to be a mixture of four components (Mazomenos and Haniotakis, 1981). Two of these components were isolated from the female rectal glands and the other two from female volatiles trapped by a total condensation cold trap operating with liquid nitrogen (Haniotakis et al., 1977).

Baker et al. (1980) identified the major component as 1,7-dioxaspiro[5.5]undecane (I). Mazomenos et al. (1981) identified the same component



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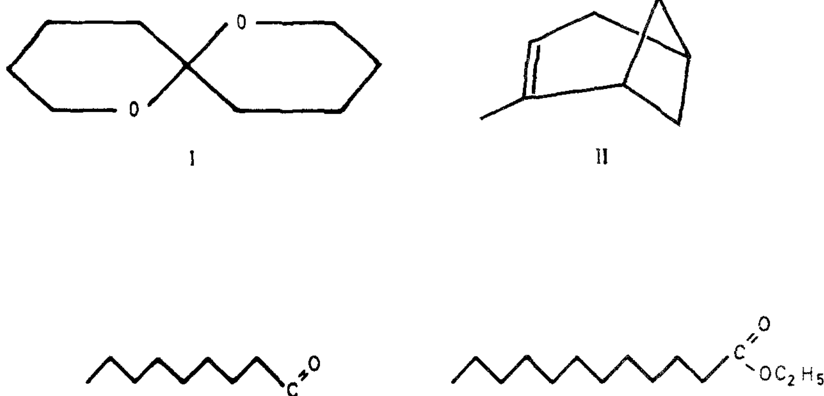


FIG. 1. Chemical structures of the sex pheromone components of female olive fruit fly, *Dacus oleae* Gmel.

plus the three secondary ones as α-pinene (II), *n*-nonanal (III), and ethyl-dodecanoate (IV). The chemical structures of all four components are shown in Fig. 1.

Laboratory and field cage bioassays showed that all components attract males. Component I is more attractive than the other three. The complete mixture is more attractive than component I (Mazomenos and Haniotakis, 1981).

Rossi et al. (1978) and Gariboldi et al. (1982) reported that the components *p*-cymene and (*Z*)- and (*E*)-6-nonen-l-ol, which were isolated from *D. oleae*, showed biological activity. However, our laboratory bioassays with these components revealed no such activity. Jones et al. (1983) tested these same components in the field and observed no fly activity. The same authors tested (*E*)- and (*Z*)-6-nonen-l-ol in combination with component I and found that (*E*)-6-nonen-l-ol depressed male catch while (*Z*)-6-nonen-l-ol had no significant effects.

In the present study we tested the synthetic pheromone components individually and in combinations in laboratory and field tests and compared them with concentrated ether extract of virgin female flies.

## METHODS AND MATERIALS

*Insects Used.* The insects used were obtained from a colony maintained at the Entomology Laboratory, N.R.C. "Democritos," Athens, Greece, on artificial diet for about 15 generations (Tsitsipis, 1977). Flies 24 hr postemergence

were separated according to sex and maintained in screen cages 270 $cm^3$ in artificial light (3000 lux intensity) and a 12:12 hr light–dark regime. Temperature was 25 ± 2°C and relative humidity 65 ± 5%.

*Pheromone Collection.* Pheromone was collected from 4- to-6-day-old virgin females by extracting the whole female body in ether for 24 hr. The extract was concentrated and stored at − 15°C until use (Mazomenos and Haniotakis, 1981).

*Synthetic Chemicals.* Component I, 1,7-dioxaspiro[5.5]undecane, was supplied by Vioryl Chemical Co. (Terma Kato Kifissia, Attikis, Greece) and was 99% pure. α-Pinene, *n*-nonanal, and ethyl-dodecanoate, components II, III, and IV, respectively, were purchased from Fluka AG, Chemical Co., Buchs SG, and were 95–98% pure. The chemicals were used without further purification.

*Laboratory Bioassays.* Bioassays were conducted in a screen cage as described by Mazomenos and Haniotakis (1981). The components to be tested were dissolved in hexane at concentrations of 6, 2, 0.6, and 2 μg/100 μl for I, II, III, and IV, respectively. These concentrations were found in preliminary tests to give optimum male response. Combinations of components were prepared similarly. Aliquots of 100 μl were poured on 7.5 $cm^2$ sections of Whatman No. 1 filter paper for each test. The solvent was allowed to evaporate before the paper was introduced into the insect cage. The number of males visiting the paper was recorded for 10 min. Each experiment was repeated four times on four different days. Filter paper with the same volume of hexane was used as control. Bioassays were conducted, one per day, during the last 2 hr of the photophase, the period with the highest mating activity (Zervas, 1982).

*Field Experiments.* During 1980, comparative male attraction studies were conducted between the complete synthetic pheromone mixture and the natural pheromone crude extract, between the major pheromone component and its various combinations with the secondary components, as well as between some combinations of secondary components alone. Yellow sticky posterboard rectangles, 15 × 20 cm, were used as traps and 1 ml polyethylene vials as pheromone dispensers (Mazomenos et al., 1983). Synthetic chemicals or their mixtures were dissolved in hexane. Concentrations of the major component tested ranged from 1 to 15 mg. When secondary components were added, the ratio of 3:1:0.3:1 was observed for components I, II, III, and IV, respectively. This is the ratio of components found in natural pheromone mixture (Mazomenos and Haniotakis, 1981).

The experimental design was either the Latin square, with traps placed in every other tree at distances of about 20 m (experiment 1 of Table 1), or the randomized block (experiment 2 of Table 1 and Figure 2 at Amarousion), or completely randomized with trap distances of about 100 m (experiment at Spata). Traps were inspected and cleaned once per week. Pheromone dispensers were not replaced throughout the experiments because of their long residual activity

TABLE 1. NUMBER OF MALE OLIVE FRUIT FLIES RESPONDING TO SYNTHETIC SEX PHEROMONE COMPONENTS AND THEIR BLEND IN LAB BIOASSAYS[a]

| Attractant | Concentration (μg) | Male response[b] |
|---|---|---|
| I | 6 | 27.5c |
| II | 2 | 10.5b |
| III | 0.6 | 11.3b |
| IV | 2 | 7.8b |
| I + II + III + IV | 6 + 2 + 0.6 + 2 | 37.3d |
| Blank | | 3.3a |

[a] Means of four replicates.
[b] Means followed by same letter are not significantly different, Duncan's multiple-range test, $P = 0.05$.

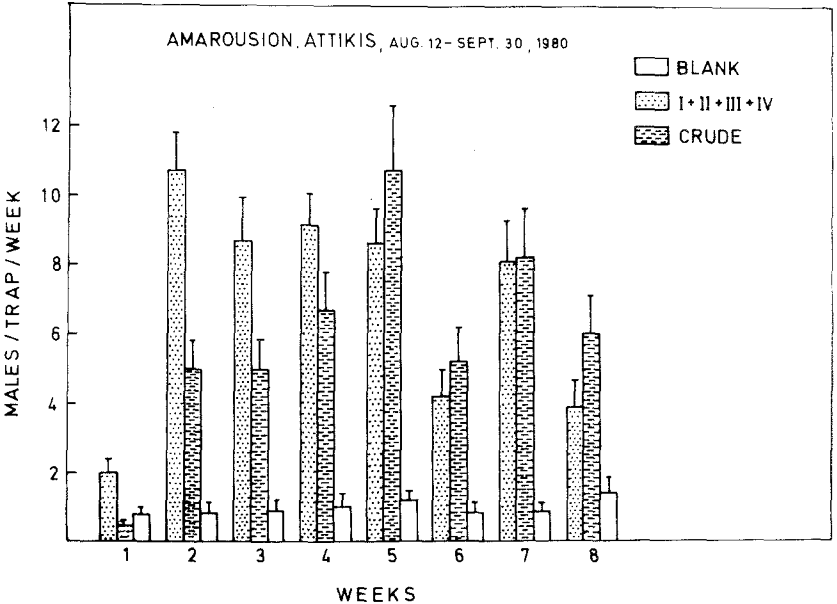


FIG. 2. Number of male olive fruit flies per trap per week captured by yellow sticky traps baited with polyethylene vials containing 400 FE of female whole body crude extract or 1 mg of the major component plus the appropriate concentrations of the other components of the blend. Means of 10 traps/treatment. Vertical lines above bars indicate standard deviations.

(Mazomenos et al., 1983). Tests relied on natural insect populations except during the period with low population densities and no wild male response to pheromones, between mid-May and the end of July (Haniotakis et al., 1982), when releases of artificially reared insects were made at weekly intervals.

During 1981, comparative male attraction studies were conducted between all four synthetic pheromone components individually and in all possible combinations with component (I). In these tests rubber septa were used as dispensers charged with 5 mg for individual component tests. For combinations, the above-mentioned ratio of components found in natural mixtures was observed. The same type of traps were used at distances of about 20 m. Traps were inspected, cleaned, rebaited, and rotated daily. Field experiment data were transformed to $\log(x + 1)$ prior to statistical analysis. Means comparisons were made with Duncan's multiple-range test.

## RESULTS AND DISCUSSION

*Laboratory Tests.* Male attraction to synthetic sex pheromone components and the complete mixture is shown in Table 1. Component I showed the major attraction, while attraction of the other three components was at lower levels. The number of males attracted to the complete pheromone mixture was higher than that to component I. Actually component I attracted 73.2% of the males attracted by the mixture. The above results coincide with those obtained from similar tests with the natural pheromone components (Mazomenos and Haniotakis, 1981), except in the case of component IV in which the natural product appears to be more active than the synthetic one.

*Field Tests.* Male olive fly attraction to various combinations and different concentrations of synthetic pheromone components and crude virgin female whole body ether extract at different periods of the year is shown in Table 2. In experiment 1, although no significant differences were found between treatments, the results were similar to the case of laboratory bioassays, i.e., combination of the major component I with the secondary components was more attractive than I alone. In addition, synthetic component I and its mixtures were more attractive than crude female extract in the concentration used.

In experiment 2, similar results were found, i.e., combination of component I with the other components, except IV, is more attractive than I alone. In addition, it can be seen that various combinations of the secondary components in the absence of I were not attractive.

Comparative male attraction studies between crude female extracts at a concentration of 400 female equivalents (FE), which contain approx. 1, 0.3, 0.1, 0.3 mg of components I, II, III, and IV, respectively, and a complete mixture of synthetic components at the above concentrations were carried out. Figure 2

TABLE 2. NUMBER OF MALE OLIVE FRUIT FLIES CAPTURED BY YELLOW POSTERBOARD STICKY TRAPS BAITED WITH POLYETHYLENE VIALS CONTAINING VIRGIN FEMALE CRUDE EXTRACT OR DIFFERENT COMBINATIONS OF SYNTHETIC PHEROMONE COMPONENTS; AMAROUSION, ATTIKIS, GREECE[a]

| Attractant | Concentration (mg) | Total males captured | Mean/trap/week[b] |
|---|---|---|---|
| Experiment 1 | | | |
| Crude | 400 FE | 207 | 4.6 |
| I | 1 | 353 | 7.8 |
| I + IV | 1 + 0.33 | 326 | 7.2 |
| I + II + III | 1 + 0.33 + 0.1 | 375 | 8.4 |
| I + II + III + IV | 1 + 0.33 + 0.1 + 0.33 | 395 | 8.8 |
| Experiment 2 | | | |
| I | 15 | 1903 | 90.6bc |
| I + II | 15 + 5 | 2510 | 119.6c |
| I + III | 15 + 1.5 | 2046 | 97.6bc |
| I + IV | 15 + 5 | 1727 | 82.3b |
| II + IV | 5 + 5 | 256 | 11.7a |
| III + IV | 5 + 5 | 280 | 13.3a |
| Blank | | 528 | 25.1a |

[a] Means of five traps/treatment (exper. 1, May 5–July 15, 1980) and three traps/treatment (exper. 2, Sept. 2–Nov. 25, 1980).
[b] Means followed by the same letter within the same experiment are not significantly different. Duncan's multiple-range test, $P = 0.05$.

shows the results. Synthetic pheromone mixture was more attractive than crude extract during the first four weeks of the experiment while during the remaining four weeks male catches were in favor of the crude extract. It seems that the evaporation rates of the two kinds of attractants were different.

In another field test with polyethylene vials as dispensers and distances between traps over 100 m the synthetic major pheromone component I at a concentration of 15 mg was compared to the complete synthetic mixture. The concentration of component I in the mixture was also 15 mg. The results are shown in Figure 3. Although not statistically different, the complete mixture attracted more males than component I alone during all 12 weeks of the experiment except of one (week 9). It should be noted that environmental temperatures dropped after the 8th week. This may have had a different effect on the evaporation rate of the different components of the mixture which resulted in a change of the existing ratio. A change of the original ratio due to different evaporation rates of the components should also be taken into consideration.

In one additional field experiment, comparative male attraction studies were conducted between all four components individually and all possible combinations of component I with the other three. In this experiment rubber septa were

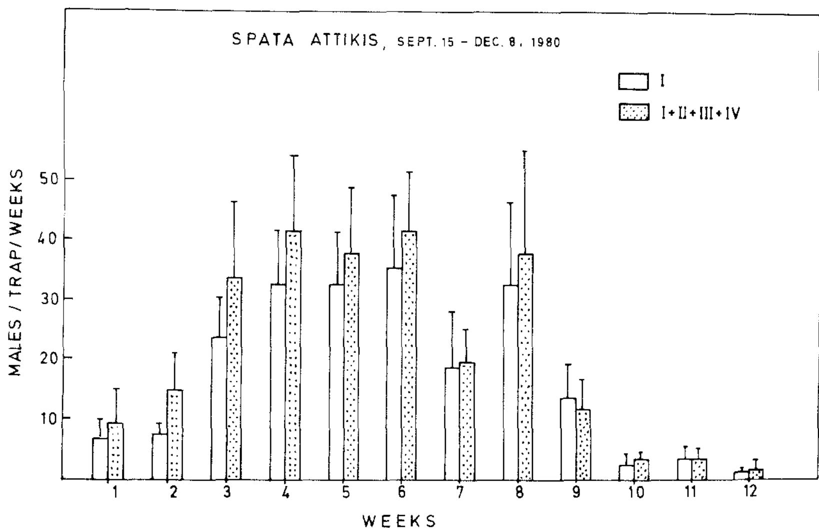


FIG. 3. Number of male olive fruit flies per trap per week captured by yellow sticky traps baited with polyethylene vials containing 15 mg of the major pheromone component or 15 mg of the major pheromone component plus the appropriate concentrations of the other components of the blend. Means of 10 traps/treatment. Vertical lines above bars indicate standard deviations.

used as dispensers. The results are shown in Table 3. As in laboratory bioassays, component I attracted also the highest number of males. In this field experiment, however, components II, III, and IV showed no significant attraction when tested individually. Furthermore, combinations of component I with one or more of the secondary components did not increase male attraction as was the case both in laboratory bioassays and in field experiments with polyethylene dispensers. It seems that the type of dispenser, i.e., the evaporation rate of individual components and subsequently their resulting ratio as they come out of the dispenser, has a decisive effect on male attraction. If this is the case, it is possible that ratio of the pheromone components as they come out of the polyethylene dispenser is suboptimal and that further increase of the biological activity of the complete pheromone mixture may be possible by appropriate formulation.

Electroantennogram (EAG) studies showed that all four pheromone components were detected by both sexes of laboratory-reared and wild insects (van der Pers et al., 1984). Components I and III elicited higher EAG response than components II and IV. These results coincide with our findings in laboratory bioassays (Table 1). Also of interest is the observation that components I and III are detected by independent sensory systems of the insect antennae.

In conclusion, even though component I and the complete mixture are attractive enough to males to be useful for practical applications, further studies

TABLE 3. NUMBER OF MALE OLIVE FRUIT FLIES CAPTURED BY YELLOW STICKY TRAPS BAITED WITH RUBBER SEPTA CONTAINING SYNTHETIC SEX PHEROMONE COMPONENTS SEPARATELY OR COMBINATIONS OF COMPONENT I WITH SECONDARY COMPONENTS[a]

| Attractant | Concentration (mg) | Total males captured | Mean/trap/day[b] |
|---|---|---|---|
| I | 5 | 641 | 23.7c |
| II | 5 | 71 | 2.6a |
| III | 5 | 121 | 4.5a |
| IV | 5 | 45 | 1.7a |
| I + II | 5 + 1.7 | 636 | 23.5c |
| I + III | 5 + 0.5 | 546 | 20.2c |
| I + IV | 5 + 1.7 | 513 | 19.0bc |
| I + II + III | 5 + 1.7 + 0.5 | 587 | 21.7c |
| I + II + IV | 5 + 1.7 + 1.7 | 331 | 12.3b |
| I + III + IV | 5 + 0.5 + 1.7 | 487 | 18.0bc |
| I + II + III + IV | 5 + 1.7 + 0.5 + 1.7 | 568 | 21.0c |
| Blank | | 31 | 1.2a |

[a] Means of three traps/treatment × 9 repetitions, between Aug. 24 and Sept. 4, 1981; Amarousion, Attikis, Greece.
[b] Means followed by the same letter are not significantly different. Duncan's multiple-range test, $P = 0.05$.

are needed to understand the mode of action and function of this pheromone system and to increase the activity of the synthetic pheromone and consequently its potential for practical use.

*Acknowledgments*—This work was partially supported by the Greek Dept. of Agriculture. The authors would like to thank Mr. A. Ioannou and N. Liopas for technical assistance, Mrs. Filipopoulou for making the drawings, and A. Kanousi for typing the manuscript.

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Journal of Chemical Ecology, Vol. 11, No. 3, 1985

# ERRATUM

## ATTRACTION OF BARK BEETLES (COLEOPTERA: SCOLYTIDAE) TO A PHEROMONE TRAP
### Experiment and Mathematical Models

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Page 728, first paragraph under RESULTS, should read: ". . . and the origin (color) of all except one on the last day, could be determined."

REFERENCE

HELLAND, I.S., HOFF, J.M., and ANDERBRANT, O. 1984. *J. Chem. Ecol.* 10:723-752.



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# ALARM SUBSTANCES OF THE STINGLESS BEE, *Trigona silvestriana*

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(Received June 24, 1983; accepted July 25, 1984)

**Abstract**—2-Nonanol, 2-heptanol, octyl decanoate, and octyl octanoate were identified from the heads of *Trigona silvestriana* workers. When presented at the nest, 2-nonanol, 2-heptanol, and the mixture of the four compounds elicited angular flights, landing, and buzzing of guard bees. Octyl octanoate elicited a weaker response. No response was given to octyl decanoate, to the ether solvent, or to the control volatile, vanillin.

**Key Words**—Alarm substances, nest defense, 2-heptanol, 2-nonanol, mandibular gland, Hymenoptera, Apidae, Meliponinae, stingless bees, *Trigona silvestriana.*

## INTRODUCTION

*Trigona silvestriana* Vachal (Apidae: Meliponinae) readily mounts a mass biting attack against large mammals in the vicinity of its nest (Johnson, 1974). Here we report that attack is released by alarm substances found in worker heads, which house the large mandibular glands (Cruz, 1962; Michener, 1974) containing the alarm pheromones of stingless bees (Blum et al., 1970; Blum and Brand, 1972; Luby et al., 1973; Keeping et al., 1982).

## METHODS AND MATERIALS

*Collection of Bees for Chemical Analysis.* In Guanacaste Prov., Costa Rica, individual worker bees were collected in a net and refrigerated. Heads of the



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torpid bees were swiftly removed with a clean razor blade and sealed with a Microflame torch into glass ampoules containing dichloromethane while the bottom of the ampoule remained frozen by immersion in liquid nitrogen.

*Sample Analysis.* The sealed glass ampoules were cooled and opened. A sample of the solvent over the bee heads was analyzed by GC and GC-MS. In addition, heads were crushed in the storage solvent and that solution analyzed. There was some increase in peak height and an increase in components with retention times longer than 18 min (Varian 3700 programing conditions: 8-min hold at 50°C, 50–270°C at 10°/min). Gas chromatographic analyses were conducted on either a Varian 3700 equipped with a 6-ft column with OV-17 as the stationary liquid phase or on a Hewlett-Packard 5830A equipped with a 20.3-m-long, and 0.3-mm-diam capillary column coated with SE-52 and with a 20.0-m-long, and 0.3-mm-diam capillary column treated with barium carbonate and coated with Silar 9CP. The programing conditions for the Hewlett-Packard 5830A were:3-min hold at 50°C, 50–250°C at 3°/min. GC-MS data were collected on a Hewlett-Packard 5985B GC-MS system, operated under standard Autotune conditions.

The earliest eluting peak (12.5% of the volatiles) was identified as 2-heptanol by comparison of GC retention times, coinjection of a known sample and the extract, and by comparison of the mass spectra. The next peak (12.7% of the volatiles) was identified in like manner as 2-nonanol. The next two peaks (23.9% and 10.0% of the volatiles) were suspected to be the octyl (large peak at 112 *m/z*) esters of octanoic acid (base peak at 145 *m/z*) and of decanoic acid (base peak at 173 *m/z*), respectively. The two esters were synthesized from the corresponding acid chloride and 1-octanol and were found to match in retention times, by coinjection and by comparison of the mass spectra. See Table 1 for retention times and mass spectral data.

To provide further confirmation of the identity of the two alcohols and the two esters, chemical ionization (methane) mass spectra were obtained. The alcohols characteristically gave a peak one mass unit less than the molecular weight, 115 for 2-heptanol and 143 for 2-nonanol. The base peak for 2-nonanol was 127, which corresponds to the ion produced by protonation of the alkene(s) formed by dehydration of the alcohol. Unfortunately, the mass range used for the set of experiments was 100–400 which missed the 99 peak for 2-heptanol. Octyl octanoate gave a base peak of 145 (octanoic acid + $H^+$) and a protonated molecular ion at 257 (69%). Octyl decanoate gave a base peak of 173 (decanoic acid + $H^+$) and a protonated molecular ion at 285 (58%).

No attempt was made to identify one component (23.4% of the volatiles) which eluted after the above two esters since the higher boiling compounds have not proven to be active as alarm pheromones. At the suggestion of a reviewer, chromatograms were obtained using a Silar 9CP column (McReynolds numbers 489, 725, 631, 913, 778) which differs considerably in retention behavior as

TABLE 1. RETENTION TIMES[a] AND MASS SPECTRAL DATA[b]

| Component | Retention time (min) | Base peak | Next three most abundant peaks and percentages | | |
|---|---|---|---|---|---|
| 2-Heptanol | 3.7 | 45 | 69(37) | 55(21) | 43(17) |
| 2-Heptanol[c] | 3.8 | 45 | 55(28) | 83(22) | 43(14) |
| 2-Nonanol | 9.8 | 45 | 69(57) | 84(35) | 55(31) |
| 2-Nonanol[c] | 10.2 | 45 | 69(28) | 55(18) | 43(16) |
| Octyl octanoate | 30.5 | 145 | 112(61) | 83(46) | 57(45) |
| Octyl octanoate[c] | 30.8 | 145 | 112(52) | 57(52) | 83(43) |
| Octyl decanoate | 35.6 | 173 | 112(92) | 83(65) | 84(57) |
| Octyl decanoate[c] | 36.5[d] | 43 | 173(96) | 51(81) | 112(70) |

[a] As observed using the H-P 5830A system and the SE-52 column.
[b] There is some variability in these data since spectra of knowns were not necessarily taken on the same day as the spectra of the components in the extract. The general pattern of the spectrum of a known and of the spectrum of a component were similar.
[c] Known compound.
[d] Matched with peak suspected to be octyl decanoate in a run for which the retention time of octyl octanoate was 31.2.

compared to the SE-52 column (McReynolds numbers 32, 72, 65, 98, 67). The point of the experiments was to determine if some of the materials which eluted from the SE-52 column after the two esters would elute earlier from a column of vastly different polarity. The results were that only the previously identified compounds eluted early and in the same order. As before, the compounds were identified by coinjection of a known compound and the extract. The remainder of the volatiles were components with retention times longer than 40 min (HP-5830A programing conditions) or components of shorter retention times and individual percentages of 3% or less not detected using the HP GC-MS system but which were detected using the HP-5930A GC system.

The quantification of the components was accomplished by spiking each of 11 samples (one bee head per vial) with a known amount of 2-tridecanone. The retention time of 2-tridecanone falls between those of 2-nonanol and octyl octanoate. A total of 25 injections were done. Since there was considerable variation from bee to bee, the results are reported as averages and simple deviations from the averages. The values, per bee head, are: 2-heptanol, 4.7 $\pm$ 2.2 $\mu$g; 2-nonanol, 7.3 $\pm$ 2.8 $\mu$g; octyl octanoate, 6.4 $\pm$ 2.2 $\mu$g; octyl decanoate, 5.8 $\pm$ 1.5 $\mu$g.

*Preparation of Material for Bioassay.* 2-Heptanol and 2-nonanol were commercial samples which were used without further purification. The two synthesized esters, octyl octanoate and octyl decanoate, were shown to be greater than 99% pure by GC analysis on the capillary column and by GC-MS analysis. Solutions in ether for bioassay contained either 2-heptanol (0.95 g/liter), 2-non-

anol (0.93 g/liter), octyl octanoate (1.16 g/liter), and octyl decanoate (0.83 g/liter), or all four compounds in the above quantities. Also tested in the bioassay were ether alone and vanillin in ether (1.05 g/liter). Portions of the stock solutions were sealed in glass ampoules for transportation to the assay sites and opened only for immediate use in the bioassays.

*Bioassay.* The bioassay was performed January 9–12, 1983, at a nest of *Trigona silvestriana* 4 m high on a tree in Santa Rosa National Park, Guanacaste Prov., Costa Rica.

Caution was needed in administering the tests because the bees readily swarm in one's hair and under the clothes, where they bite. The object of the tests was to investigate the bees' response to the chemicals, not to the experimenter. The experimenter slowly approached the nest and took cover behind a tree 5 m in front of the nest entrance. No trial was run if there was the slightest sign of searching or alertness by guard bees. If the bees were calm, that is, the only activity was straightforward flight in and out of the nest tube, the test chemical was presented. Behind the tree the investigator pipetted 10 $\mu$l of test solution onto a 3.5-cm$^2$ piece of filter paper suspended by a short string from a long pole of roadside grass mounted on a stick. Within 5 sec the paper was smoothly thrust to a position 25 cm slightly upwind of the entrance, and held there for 1 min.

From the post 5 m in front of the nest tree, two kinds of alarm response could be reliably discerned. The big (7 mm) black workers of *T. silvestriana* that landed on the white paper could be readily counted. In addition, the presence or absence of short, erratic flights by bees in front of the entrance could be noted. These angular flight paths presented a sharp contrast to the smooth trajectories in and out of the nest tube normally made by the bees.

The following treatments were used, in order of presentation: (1) untreated paper, (2) ether, (3) vanillin in ether, (4) octyl octanoate in ether, (5) octyl decanoate in ether, (6) 2-nonanol in ether, (7) 2-heptanol in ether, and (8) a mixture of octyl octanoate, octyl decanoate, 2-nonanol and 2-heptanol in ether. The amounts tested were small, so as to be commensurate with the amount in one bee head. Vanillin was a control to test for the possibility that the bees would respond to the sudden presentation of any volatile chemical.

During the trials of 2-nonanol, 2-heptanol, or the mixture, bees sometimes located the experimenter. If this happened, data from that trial were discarded and another test attempt was made later in the day or on the following morning, after a shower and change of clothes. At least 15 min elapsed between trials or attempts; a clean paper, string, and pole were used each time. Over the four days, the series of chemicals was presented three times.

At the end of the second day a foraging *T. silvestriana* was located 10 m from the nest. Liquid expressed from its head was tested in the same way as the other chemical substances.

## RESULTS

No response occurred in three trials to the untreated paper square, to the ether alone, or to the vanillin or octyl decanoate in ether. Bees landed on the paper or flew in angular, erratic paths when 2-nonanol, 2-heptanol, and the mixture were presented; the bees responded two out of three times when octyl octanoate was presented (Table 2). Landing was accompanied by buzzing, which could be felt along the pole when bees landed on the pole above the suspended paper.

A chi-square test of the landings allows us to reject the null hypothesis of no difference in the distribution of the 15 observed landings among the eight treatments ($\chi^2 = 26.068$, 7 *df*, $P < 0.005$). When treatments were combined into categories (controls, esters, and alcohol-containing treatments; or controls, esters, alcohols, and mixture), the differences remained significant at the $P < 0.005$ level. A Fisher exact probability test, furthermore, rejects the null hypothesis of no difference in proportion of trials with landing or erratic flight responses between the control and the experimental (bee chemical) trials ($P = 0.0005$).

The landing, flight, and buzzing responses were similar to those observed when the material from one fresh head was presented. During that 1-min test period, six landings occurred, buzzing was felt, and a large number of erratic flights were made.

Five of the nine trials of the alcohols or mixture were repeats of earlier

TABLE 2. RESPONSE OF BEES AT NEST TO TREATED PAPER SQUARE PRESENTED FOR 1 MINUTE

| Treatment | Number of landings, trial | | | Total bees landing on paper[a] | Erratic flights, trial | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | 1 | 2 | 3 |
| Control | 0 | 0 | 0 | 0 | – | – | – |
| Ether | 0 | 0 | 0 | 0 | – | – | – |
| Vanillin | 0 | 0 | 0 | 0 | – | – | – |
| Octyl decanoate | 0 | 0 | 0 | 0 | – | – | – |
| Octyl octanoate | 0 | 1 | 0 | 1 | – | + | ++[b] |
| 2-Nonanol | 1 | 1 | 0 | 2 | ++ | ++ | ++ |
| 2-Heptanol | 2 | 3 | 1 | 6 | ++ | ++ | ++ |
| Mixture | 3 | 1 | 2 | 6 | ++ | ++ | ++ |

[a] A chi-square test rejected the null hypothesis of no difference in the distribution of the 15 landings among the eight treatments ($\chi^2 = 26.068$, 7 *df*, $P < 0.005$).

[b] –: no erratic flights; +: 2 bees made erratic flights; ++: >2 bees made erratic flights (i.e., too many to count).

attempts during which the bees, after first responding to the test substance, found and attacked the experimenter behind the tree.

## DISCUSSION

2-Heptanol and 2-nonanol are recognized alarm substances in bees of the family Apidae. Collins and Blum (1982, 1983) report that 2-heptanol and 2-nonanol from the sting extracts of honeybees release alarm behaviors in caged young workers. Keeping et al. (1982) found that 1-nonanol and 2-nonanol together made up 16% of the mandibular secretions of *Trigona gribodoi;* when worker heads were crushed near the nest, the bees closed the top of the entrance tube and ceased flight activity (Crewe and Fletcher, 1976). Luby et al. (1973) found significant amounts of 2-heptanol in the heads of *Trigona mexicana* and 2-heptanol and 2-nonanol in the heads of *Trigona pectoralis.* When these chemicals were wafted in front of the nest, the bees moved quickly, investigated, or attacked. 2-Heptanol and 2-nonanol also function as trail substances in *Trigona spinipes,* according to Blum (1979). Kerr et al. (1981) confirmed the trail function of 2-heptanol when, using an artificial trail of 2-heptanol drops, they led *T. spinipes* foragers to dishes of syrup the bees had visited a day earlier, but had abandoned.

Octyl octanoate and octyl decanoate are found in *T. spinipes* (Kerr et al., 1981) and are similar to the octyl hexanoate found in *Trigona fulviventris* (Johnson and Wiemer, 1982). The function of these esters is unknown. Octyl hexanoate did not appear to be an alarm substance in *T. fulviventris,* nor did it consistently act synergistically with the releaser alcohol, nerol. In *T. silvestriana,* octyl octanoate, but not octyl decanoate, elicited alarm behaviors, at least part of the time, but was not detectably synergistic with the alcohols. No function for these esters has been reported for *T. spinipes.* The esters may play some role in the marking of food sources by foraging bees; alternatively, the esters may represent material from the cuticle.

Overall, the similarity of the mandibular gland chemistry of *T. silvestriana* and *T. spinipes* is striking. The four compounds we found in *T. silvestriana* are also found in *T. spinipes,* plus 2-tridecanol, according to Kerr et al. (1981). These authors regarded the mandibular gland chemistry of *T. spinipes* as highly distinctive. This claim no longer holds, but they may be right in saying that *T. spinipes* lays a unique trail in Brazil, for *T. spinipes* is a South American species and *T. silvestriana* a Central American one.

Attraction, landing, buzzing, and angular flights are typical alarm behaviors in *Trigona* bees (cf. Blum et al., 1970; Luby et al., 1973; Johnson, 1980; Keeping et al., 1982; Johnson and Wiemer, 1982). Although mandibular movements could not be seen from a distance of 5 m, it is likely that *T. silvestriana* bees that landed on the test paper were also biting it. Biting is a prominent form

of nest defense in those *Trigona*, such as *T. silvestriana, T. corvina*, and *T. fuscipennis*, that have strong, sharp, 5-toothed mandibles and live in exposed or semiexposed nests (Johnson, 1974).

Of interest was the fact that the guard bees were more likely to locate the experimenter if 2-heptanol or 2-nonanol or the mixture had just been presented at the entrance. This suggests that the bees might be made more sensitive to provocative sights, smells, or vibrations (Free 1961) by prior exposure to alarm pheromone. Collins et al. (1980) found for honeybees a hierarchy of stages of arousal, elicited by different kinds of stimuli, from "alert" (which could be elicited by odors), through "activate" and "attract," to "culminate," in which bees actively defend the nest by biting, pulling hair, and stinging.

We propose that stingless bees also possess a hierarchy of stages of arousal in nest defense. A few guard bees, with the lowest thresholds, investigate a possibility. If they find additional stimuli, as from a moving, smelly, dark, hairy, warm animal (Free, 1961; Johnson, unpubl. data), they perform marking and other behaviors that alert other bees. These bees in turn may or may not be stimulated to arouse still others, depending upon the magnitude of the intruder problem. In this way the scale of the colony response is appropriately adjusted and maladaptive expenditure of energy is avoided. In the present study the number of bees around the filter paper was always small; only when additional stimuli from the experimenter were encountered was there sufficient provocation for an escalated attack response.

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# *Lasioderma* CHEMISTRY
## Sex Pheromone of Cigarette Beetle (*Lasioderma serricorne* F.)

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**Abstract**—A chemical study of the sex pheromone of the cigarette beetle was carried out. Seven components were isolated from active fractions of column chromatography of the female extract, and their structures were elucidated by spectroscopic evidence and confirmed by synthesis to be (4*S*,6*S*,7*S*)-4,6-dimethyl-7-hydroxynonan-3-one (serricornin) (I), 2,6-diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran (anhydroserricornin) (II), 4,6-dimethylnonan-3,7-dione (III), 4,6-dimethylnonan-3,7-diol (IV), 4,6-dimethyl-7-hydroxy-4-nonen-3-one (V), (2*S*,3*R*)-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (serricorone) (VI) and (2*S*,3*R*)-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-hydroxybutyl)-4H-pyran-4-one (serricorole) (VII).

These structural features suggested that the occurrence of these components might be related to the polyketide biosynthesis. The behavioral bioassay and EAG experiments revealed the biological role of each component in the copulatory behavior of this insect.

**Key Words**—Sex pheromone, *Lasioderma serricorne* F., cigarette beetle, Coleoptera, Anobiidae, serricornin, polyketide biosynthesis.

## INTRODUCTION

The cigarette beetle (*Lasioderma serricorne* F.) is a serious cosmopolitan pest of not only cured tobacco leaves but also of nearly all dry food stuffs. The difficulty in detecting the infestation of this pest until their population has increased beyond the economic threshold level because of their clandestine nature and the deficiency of pesticide-dependent control methods led us to investigate



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the sex pheromone of this insect. The goal of this investigation was the development of efficient tools for integrated pest control.

The existence of the sex pheromone produced by female cigarette beetles has been previously reported (Burkholder, 1970). A simple quantitative laboratory bioassay technique has also been developed (Coffelt and Burkholder, 1972). It was revealed that the male responses to the sex pheromone consisted of a sequence of copulatory behaviors such as an antennal elevation with leg extension, rapid locomotion to the pheromone source, and copulatory attempts with other test males.

No chemical studies had been reported when our study on the sex pheromone of this insect started. The structural elucidation and the synthesis of serricornin (Chuman et al., 1979a,b), determination of the absolute configuration of serricornin by stereoselective syntheses (Chuman et al., 1981; K. Mori et al., 1981, 1982; M. Mori et al., 1982a,b), the structure–pheromone activity relationship of serricornin (Chuman et al., 1982a,b) has already been reported. Recently, the structural elucidations of serricorone and serricorole were also reported (Chuman et al., 1983).

In this article, we wish to describe the isolation, structural elucidation, syntheses, and sex pheromone activity of these components in detail, although some parts of this study have already been reported in the short communications, and to discuss the occurrence of the sex pheromone components related to the polyketide biosynthesis (Figure 1).

FIG. 1. Structures of the sex pheromone components.

## METHODS AND MATERIALS

Cigarette beetles were reared on corn flour containing 5% brewer's yeast powder at 28°C and 60% relative humidity with a 1:1 light–dark photoperiod. Virgin females for extraction and the males for bioassay and EAG experiments were sexed at the pupal stage by the tail characteristic. The pheromone activity was estimated by behavioral bioassy and EAG experiments as described previously (Chuman et al., 1982a,b).

### *Analysis*

IR spectra refer to films and were determined on a Jasco IRA-1 spectrometer. The 100-MHz FT-PMR and CMR spectra were recorded in $CDCl_3$ with TMS as an internal standard on a JEOL-FX-100 spectrometer unless otherwise mentioned. Optical rotations were measured on a Jasco DIP-4 polarimeter. Capillary GC analyses were performed on a Shimadzu Minim-1 GC using OV-101 (30 m, 0.25 mm ID). Preparative GC was performed on a Hitachi 063 GC using OV-101 (1 m, 3 mm ID). The GC-MS analyses were carried out on JEOL-D-300, Hitachi 50GC, and Hitachi M80 mass spectrometers.

### *Preexamination of Pheromone Isolation*

To examine the chemical properties of the pheromone prior to the large scale isolation, about 100 virgin females were extracted with hexane. The extract could elicit the strong sex pheromone activity on attractiveness and sex stimulation for the male. When the extract was reacted with 2,4-dinitrophenylhydrazine or with acetic anhydride and pyridine, nearly all the activity disappeared. The activity could be recovered by hydrolysis of the acetylated material with 3% KOH–methanol. Column fractionation on silicic acid with hexane–ether mixtures yielded a low-polarity fraction, eluted with 2–5% ether/hexane, that was very active and a high-polarity fraction, eluted with 100% ether, that was somewhat active. The activity of the low-polarity fraction was relatively stable to alkali, but unstable to acid. It was also unstable to heat as demonstrated by loss of activity on attempted preparative GC purification. This instability to preparative GC purification could be avoided by acetylation of the 2–5% ether/hexane fraction from column chromatography.

### *Large-Scale Isolation*

The 260,000 cigarette beetles (mixed population, F/M ratio = 1:1) were extracted with hexane, and the crude extract was chromatographed on a silicic acid column with hexane–ether mixtures as a solvent to furnish two active fractions, which corresponded to the 2–5% ether/hexane and 100% ether fractions. The 2–5% ether/hexane fraction, which was the main active fraction, was

acetylated with acetic anhydride and pyridine. Final purification by preparative GC gave compound I (3.1 mg) as a main component, compound III (1.0 mg), and compound V (1.8 mg) from the acetylated 2–5% ether/hexane fraction, and compound VI (1.2 mg) and compound VII (0.7 mg) from the 100% ether fraction. Compound II and compound IV were detected as minor components in the 2–5% ether/hexane fraction by GC-MS analysis.

*Syntheses of Components* (Fig. 2).

*4,6-Dimethyl-7-hydroxy-nonan-3-one (Serricornin) (I) and 2,6-Diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran (Anhydroserricornin) (II) (Figure 2).* Crude serricornin could be obtained by the method described previously (Ono et al., 1980). The crude serricornin (100 g) was refluxed in benzene in the presence of TsOH and distilled to furnish pure 2,6-diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran (anhydroserricornin, II, 54 g) in 60% yield. Boiling point 37–38° C/1 mm Hg, MS ($m/z$): 168($M^+$, 38), 41(46), 43(54), 55(58), 57(42), 69(42), 86(31), 99(100), 111(12), 125(19), 139(15), 153(4); IR ($cm^{-1}$, film): 2970(s), 2930(s), 2840(s), 1682(s), 1460(s), 1380(s), 1350(m), 1270(s), 1230(s), 1170(s), 1050(s), 983(s); PMR ($CDCl_3$, $\delta$ TMS): 0.8–1.0 (9H, m), 1.2–1.8 (5H, m), 1.56 (3H, bds), 2.0 (2H, q), 3.2–3.6 (1H, m); CMR ($CDCl_3$, $\delta$, TMS): 9.5 (t), 10.6 (c), 12.2 (c,t), 13.5 (c,t), 17.5 (c), 17.7 (t), 23,6(cx2), 23.9 (t), 25.4 (t), 30.1 (c), 31.4 (t), 35.7 (c), 35.9 (t), 79.0 (c), 80.7 (t), 98.6 (c), 99.5 (t), 148.4 (c), 148.9 (t); multiplicity of completely decoupled CMR signals was due to the coexistence of $C_{2,3\text{-}cis}$-(c) and $C_{2,3\text{-}trans}$-(t) isomers.

Treatment of pure anhydroserricornin (II, 54 g) with iso-PrOH (600 ml) and water (200 ml) in the presence of TsOH (20 g) at 35°C for 6 hr gave 4,6-dimethyl-7-hydroxy-nonan-3-one (serricornin, I) (51.9 g) with anhydroserricornin (II, 7.1 g). GC analysis showed that this stereoisomeric mixture of serricornin was composed of (4*S**, 6*S**, 7*S**)[1] isomer (33%), (*S**, *S**, *R**)[2] isomer (9%), (*S**, *R**, *S**) isomer (48%), and (*S**, *R**, *R**) isomer (10%). The assignments of the stereochemistries were established by the complete decoupled CMR data of each acetate of serricornin stereoisomers (Table 1).

Column chromatography of the stereoisomeric mixture of serricornin on silica gel furnished pure (*S***R***S**)- and (*S***R***R**) serricornins. Pure (*S***S***S**) and (*S***S***R**) serricornins could be obtained by the $C_4$ epimerization of (*S***R***R**) and (*S***R***S**) serricornins, respectively, because the chromatographic behaviors of (*S***S***S**) and (*S***S***R**) serricornins were very close and the direct separation on the column failed.

[1] A stereochemical sign with asterisks such as (4*S**,6*S**,7*S**) means the racemate of the respective enantiomers.

[2] Stereochemical signs such as (*SSS*), (*SSR*), (*SRS*), and (*SRR*) mean (4*S*,6*S*,7*S*), (4*S*,6*S*,7*R*), (4*S*,6*R*,7*S*) and (4*S*,6*R*,7R), respectively.

FIG. 2. Synthesis of the sex pheromone components.

TABLE 1. CMR DATA OF ACETATES OF SERRICORNIN STEREOISOMERS[a]

| Structure | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | C-7 | C-8 | C-9 | C-10 | C-11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (±)– (SSS) OAC | 7.84 | 34.22 | 214.88 | 43.53 | 24.22 | 33.70 | 78.04 | 35.94 | 10.18 | 16.67 | 14.45 |
| (±)– (RSS) OAC | 7.84 | 34.28 | 214.83 | 43.35 | 24.22 | 33.70 | 77.75 | 36.39 | 10.18 | 17.32 | 14.63 |
| (±)– (SRS) OAC | 7.78 | 34.11 | 214.88 | 43.88 | 23.34 | 34.11 | 78.92 | 35.51 | 10.00 | 18.08 | 15.85 |
| (±)– (RRS) OAC | 7.84 | 34.34 | 214.94 | 43.58 | 23.28 | 33.81 | 79.09 | 35.01 | 10.06 | 16.21 | 15.33 |

[a] The previous stereochemical assignments of (*S*, *R*, *S*)- and (*R*, *R*, *S*)-serricornin described in Chuman et al. (1981a) were revised. The assignment in this article was reversed.

Among these four enantiomeric mixtures of serricornin, only the ($S^*R^*S^*$) isomer yielded a cubic crystalline material (mp 46.5–47.5°C). X-ray analysis of the crystalline material established the structure to be ($2R^*$, $3S^*$, $5R^*$, $6S^*$)-2,6-diethyl-3,5-dimethyl-2-hydroxy-tetrahydropyran, which corresponded to the hemiketal form of ($S^*R^*S^*$)-ketoalcohol form of serricornin (M. Mori et al., 1984). IR ($cm^{-1}$, KBr): 3400(s), 2970(s), 2930(s), 2870(s), 1460(s), 1400(s), 1375(s), 1265(m), 1180(s), 1150(s), 1090(s), 1070(s), 1040(m), 1000(s), 970(s), 920(s), 900(m); PMR ($CDCl_3$, $\delta$ TMS): 0.7–1.0 (12H,m), 1.2–1.8(8H,m), 3.34 (1H,ddd, $J$ = 3, 7.5, 10 Hz); CMR ($CDCl_3$, $\delta$ TMS): 7.1, 9.6, 16.4, 17.6, 25.6, 32.6, 35.0, 36.7, 37.1, 76.0, 98.2.

*4,6-Dimethylnonan-3,7-dione (III).* The mixture of serricornin (I, 3.7 g) and $CrO_3$–pyridine complex in pyridine (150 ml) was stirred for 48 hr at 10°C and poured into water. The mixture was extracted with benzene and the benzene layer was washed with 10% aq HCl to eliminate pyridine. After washing with water and drying over $Na_2SO_4$, the mixture was concentrated in vacuo to give 4,6-dimethylnonan-3,7-dione (III, 3.5 g). MS (*m/z*), 184($M^+$, 1): 41(10), 57(100), 86(40), 99(4), 127(4), 142(2), 155(1); IR ($cm^{-1}$, film): 2970(s), 2940(s), 2880(s), 1710(s), 1460(s), 1410(m), 1375(s), 1100(s), 1020(m), 975(s); PMR ($CDCl_3$, $\delta$ TMS): 1.04(6H, t, $J$ = 7 Hz), 1.07 (6H, d, $J$ = 7 Hz), 1.6 (2H, m), 2.4 (6H, m).

*4,6-Dimethylnonan-3,7-diol (IV).* A solution of serricornin (I, 5.6 g) is iso-PrOH (10 ml) was added dropwise to a stirred and ice-cooled suspension of $NaBH_4$ (0.38 g) in iso-PrOH (20 ml) and the mixture was stirred at room temperature overnight. The mixture was poured into ice-water, treated with 10% aq acetic acid, and extracted with ether. The ether layer was washed with water and dried over $Na_2SO_4$ to give 4,6-dimethylnonan-3,7-diol, (IV, 5.3 g). MS (*M/z*): 41(40), 43(40), 55(40), 57(40), 59(60), 69(40), 83(16), 86(8), 123(16), 141(4); IR ($cm^{-1}$, film): 3300(s), 2960(s), 2930(s), 2875(s), 1455(s), 1370(s), 1340(s), 1250(m), 1240(m), 1140(m), 1100(m), 1060(m), 975(s), 950(s); PMR ($CDCl_3$, $\delta$ TMS): 0.8–1.0 (12H, m), 1.2–1.7 (8H, m), 3.4 (2H, m); 3,7-Diacetoxy-4,6-dimethylnonane (diacetate of IV), MS (*m/z*): 43(100), 55(16), 69(20), 70(24), 83(10), 86(6), 103(11), 109(5), 123(11), 141(7), 152(2), 154(3).

*2,3-*cis*-2,3-Dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (Serricorone, VI).* A solution of $BF_3$–$Et_2O$ (28.4 g) in ether was added dropwise to a stirred and ice-cooled solution of 4-methylheptan-3,5-dione (iii, 28.4 g) in dry ether (100 ml) at 5–10°C. To the mixture, propionaldehyde (18.5 g) was added with stirring. After addition, the mixture was continuously stirred for 10–16 hr, subsequently poured into ice-water and extracted with ether. The ether layer was washed with $Na_2SO_3$, water, and dried over $Na_2SO_4$. The ether layer was concentrated *in vacuo,* and the residue was distilled to furnish 2,3-dihydro-3,5-dimethyl-2,6-diethyl-4H-pyran-4-one (iv) (18.8 g) in 56% yield, bp 73–82°C/3 mm Hg. Capillary GC analysis revealed that the ratio of the $C_{2,3\text{-}cis}$

and $C_{2,3\text{-}trans}$ isomers was 3:1. Pure 2,3-*cis* pyranone was obtained by column chromatography. MS ($m/z$): 182($M^+$, 33), 55(67), 57(93), 83(89), 113(100), 153(1); PMR ($CDCl_3$, $\delta$ TMS): 0.98 (3H, t, $J$ = 7 Hz), 1.03 (3H, d, $J$ = 7 Hz), 1.14 (3H, t, $J$ = 7 Hz), 1.73 (3H, s), 2.2–2.4 (3H, m), 4.12 (1H, ddd, $J$ = 8.2, 5.8, 3.1 Hz); CMR ($CDCl_3$, $\delta$ TMS): 9.1, 9.5, 9.8, 10.9, 23.6, 25.6, 42.5, 82.0 (cf. $C_{2,3\text{-}trans}$: 83.7); signals due to the carbons belonging to the carbonyls and the fully substituted double bond were not observed because of the short pulse interval used for the CMR measurement.

The mixture of the $C_{2,3\text{-}cis}$ pyranone (iv) (33.6 g) and propionic anhydride (78 g) was stirred in the presence of $ZnCl_2$ (24.9 g) for 48 hr at 40°c. After cooling, the mixture was poured into ice-water and the ether layer was separated. The ether layer was washed with saturated $NaHCO_3$ to remove excess propionic anhydride and with 10% aq $Na_2CO_3$, water and dried over $Na_2SO_4$. After concentration, the residue was dissolved in 40% aq dimethylamine–methanol (30 ml) and allowed to stand for 24 hr. After filtration, the filtrate was poured into ice-water and extracted with ether. The ether layer was washed with water and dried over $Na_2SO_4$. The ether solution was concentrated, and the residue was chromatographed on silica gel to give 2,3-*cis*-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (2,3-*cis* serricornone) (VI, 26.8 g) in 60% yield. MS ($m/z$): 238 ($M^+$, 5), 43(13), 55(27), 57(100), 69(7), 83(28), 97(8), 109(15), 112(9), 113(30), 124(18), 139(4), 153(7), 182(53), 183(7); IR ($cm^{-1}$, film): 2950(s), 2930(s), 2860(s), 1710(s), 1660(s), 1605(s), 1455(m), 1370(m), 1340(m), 1200(m), 1135(w), 1120(m), 1045(m); PMR ($CDCl_3$, $\delta$ TMS): 0.99 (3H, t, $J$ = 7.8 Hz), 1.03 (3H, d, $J$ = 7.8 Hz), 1.07 (3H, d, $J$ = 7.1 Hz), 1.30 (3H, d, $J$ = 6.9 Hz), 1.33 (3H, d, $J$ = 6.9 Hz), 1.80 (3H, s), 1.4–1.8 (2H, m), 2.4 (3H, m), 3.70 (1H, q, $J$ = 6.6 Hz), 4.15 (1H, m); CMR ($CDCl_3$ $\delta$, TMS): 7.8, 9.5, 9.7, 12.7, 12.9, 23.3, 23.6, 33.9, 42.7, 49.1, 49.5, 8.27, 83.0 (cf. $C_{2,3\text{-}trans}$ isomer: 84.3); signals due to the carbons belonging to the carbonyls and the double bond were not observed.

*2,3-cis-2,3-Dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-hydroxybutyl)-4H-pyran-4-one (serricorole, VII).* To an ice-cooled suspension of $NaBH_4$ (0.38 g) in EtOH (20 ml), a solution of 2,3-*cis*-serricorone (VI, 2.38 g) in EtOH (5 ml) was added without stirring, and the resulting solution was allowed to stand for 5–6 hr. The solution was added to ice-water and extracted with ether. The ether layer was washed with water and dried over $Na_2SO_4$. Concentration in vacuo gave 2,3-*cis*-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-hydroxybutyl)-4H-pyran-4-one (serricorole VII, 1.6 g). MS ($m/z$): 43(16), 55(16), 57(49), 59(67), 69(23), 70(30), 83(46), 96(15), 97(18), 109(38), 111(13), 112(99), 113(100), 117(15), 124(37), 141(17), 153(28), 182(80); IR ($cm^{-1}$, film): 3400(bds), 2950(s), 2920(s), 2870(s), 1650(s), 1640(s), 1600(s), 1455(s), 1370(s), 1190(m), 1150(m), 1130(m), 1060(m), 1030(m), 970(m); PMR ($CDCl_3$, $\delta$ TMS): 0.9–1.2 (12H, m), 1.4–1.6 (4H, m), 1.75 (3H, s), 2.21 (2H, m), 2.82 (1H, m), 3.5–4.2

(2H, m). 2,3-*cis*-2,3-Dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-acetoxybutyl)-4H-pyran-4-one (acetate of 7), MS ($m/z$): 282($M^+$, 4), 43(67), 57(15), 69(10), 70(12), 83(52), 97(58), 101(26), 109(10), 113(16), 124(24), 125(11), 152(53), 153(100), 171(22), 182(76), 193(66), 222(59).

## RESULTS AND DISCUSSION

### *Structural Elucidation of the Components*

*(4S, 6S, 7S)-4,6-Dimethyl-7-hydroxynonan-3-one (Serricornin, I).* Compound I was isolated from the 2–5% ether/hexane fraction of column chromatography and purified by preparative GC after acetylation. $[\alpha]_D^{23}$-17.7, $[\alpha]_{546}$-19.7, $[\alpha]_{435}$-36.8, $[\alpha]_{365}$-70.3 (c = 0.155, hexane), MS ($m/z$): 168 ($M^+$-$CH_3COOH$, 8), 43(100), 55(24), 57(71), 69(39), 70(20), 83(27), 86(64), 99(13), 111(27), 127(4), 128(5), 139(20), 153(1), 157(20); IR ($cm^{-1}$, film): 2960(s), 2940(s), 2870(s), 1735(s), 1715(s), 1460(m), 1370(m), 1240(s), 1100(m), 1020(m), 960(m), 890(m); PMR ($CDCl_3$, $\delta$ TMS): 0.86 (3H, t, $J$ = 7 Hz), 0.89 (3H, d, $J$ = 7 Hz), 1.05 (3H, t, $J$ = 7 Hz), 1.08 (3H, d, $J$ = 7 Hz), 1.43 (2H, m), 1.60 (1H, m), 1.66 (2H, m), 2.06 (3H, s), 2.44 (2H, q, $J$ = 7 Hz), 2.63 (1H, m), 4.78 (1H, m), irradiation at 1.43, 1.60, 2.44, and 2.63 simplified the t at 0.86 to s, the t at 0.89 to s, the t at 1.05 to s, and the d at 1.08 to s, respectively; CMR ($CDCl_3$, $\delta$ TMS): 7.84, 10.18, 14.45, 16.67, 21.06, 24.22, 33.70, 34.22, 35.98, 43.53, 78.04, 170.88, 214.88.

Structural elucidation of serricornin (compound I) as its acetate form by spectroscopic evidence and its synthesis has already been reported (Chuman et al., 1979a,b). The absolute configurations of the three chiral centers at $C_4$, $C_6$, and $C_7$ in the serricornin molecule were established to be (4*S*,6*S*,7*S*) by several synthetic studies of serricornin stereoisomers (Chuman et al., 1981a; K. Mori et al., 1981; M. Mori et al., 1982a) and (4*S*,6*S*,7*S*)-serricornin, the natural isomer (K. Mori et al., 1982; M. Mori et al., 1982b; Mori and Watanabe, 1984).

In addition to the stereochemistry, the serricornin molecule is also of interest because it can exist in both the acyclic chain and cyclic hemiketal forms. This assumption was strongly supported by the actual occurrence of the hemiketal form of ($S^*R^*S^*$)-serricornin as a crystalline material. Recently, detailed assignments of high-field 500-MHz PMR spectral data revealed that natural (*SSS*)-serricornin existed at equilibrium between the acyclic keto alcohol form and cyclic hemiketal form with a ratio of 1:2.5 in $C_6D_6$ solution. The 500-MHz PMR studies of the serricornin stereoisomers also indicated that nonnatural isomers, ($S^*R^*S^*$)- and ($S^*R^*R^*$)-serricornins existed predominantly in the hemiketal form and the keto alcohol form, respectively, at equilibrium, but ($S^*S^*R^*$)-serricornin existed at equilibrium in the same ratio as that of

FIG. 3. Equilibrium between the acyclic keto alcohol and the cyclic hemiketal forms of serricornin.

($S^*S^*S^*$)-serricornin (Figure 3). These differences of the equilibrium relationships among serricornin stereoisomers were considered to be due to the stereochemical relationship between $C_4$ and $C_6$ methyls in the hemiketal molecule. Although this equilibrium between the keto alcohol and the hemiketal forms seems to be closely related to the occurrence of the sex pheromone activity of serricornin, it remained unknown whether the active form of serricornin was the acyclic keto alcohol form or the cyclic hemiketal form and whether both were required with some ratio in the copulatory communication of the cigarette beetle. Judging from the inactivity of anhydroserricornin (II), which formed easily by dehydration of serricornin, it is possible that the structural conversion between keto alcohol–hemiketal and anhydroserricornin has a regulatory function of the occurrence and disappearance of the pheromone activity (Figure 4).

*4,6-Dimethylnonan-3,7-dione (III).* Compound III was isolated from the acetylated 2–5% ether/hexane fraction and purified by preparative GC. $[\alpha]_D^{23}$ +6.0, $[\alpha]_{545}$ +0, $[\alpha]_{436}$ +0, $[\alpha]_{365}$-34.0 (c = 0.05, hexane); MS (m/z): 184 ($M^+$, 1), 41(10), 57(100), 86(49), 99(6), 127(11), 142(5), 155(3); IR($cm^{-1}$, film): 2790(s), 2925(S), 2870(s), 1710(s), 1460(m), 1375(m), 1100(m),

Keto-alcohol serricornin — Hemiketal serricornin — Anhydro serricornin

ACTIVE — INACTIVE

FIG. 4. Structural conversion of the serricornin molecule.

1020(w), 965(m); PMR ($CDCl_3$, $\delta$ TMS): 1.04 (6H, t, $J$ = 7 Hz), 1.08 (6H, d, $J$ = 7 Hz), 1.66 (2H, m), 2.44 (4H, q, $J$ = 7 Hz), 2.55 (2H, m), irradiation at 2.55 simplified at t at 1.04 and the d at 1.08 to bds and the m at 1.66 to s, respectively.

Structural elucidation of 4,6-dimethyl-nonan-3,7-dione by spectroscopic evidence has already been reported (Chuman et al., 1979a).

*4,6-Dimethyl-7-hydroxy-4-nonen-3-one (V).* Compound V was isolated from the acetylated 2–5% ether/hexane fraction and purified by preparative GC. $[\alpha]_D^{23}$-24.8, $[\alpha]_{546}$-41.1, $[\alpha]_{435}$-56.7, $[\alpha]_{365}$-114.4 (c = 0.09, hexane); MS (m/z): 168($M^+$-$C_2H_5COH$, 2), 43(100), 57(15), 67(8), 69(8), 97(33), 109(7), 126(93), 137(24), 155(12), 166(2); IR ($cm^{-1}$, film): 2960(s), 2920(s), 2870(s), 1730(s), 1665(s), 1455(m), 1370(m), 1240(s), 1085(m), 1040(m), 1015(m), 960(m), 900(m); PMR ($CDCl_3$, $\delta$ TMS): 0.87 (3H, t, $J$ = 7 Hz), 1.02 (3H, d, $J$ = 7 Hz), 1.09 (3H, t, $J$ = 7 Hz), 1.4–2.0 (3H, m), 1.79 (3H, d, $J$ = 1.5 Hz), 2.66 (2H, q, $J$ = 7 Hz), 2.08 (3H, s), 4.78 (1H, m), 6.38 (1H, d, $J$ = 7 Hz), irradiation at 1.6, 2.66, and 6.38 simplified the t at 0.87 to s, the t at 1.09 to s, and the d at 1.79 to s, respectively.

Precise mass determination of the ions at $m/z$ 166 and 168, which were caused by a loss of $CH_3COOH$ and $C_2H_5COH$ from molecular ion, respectively, in EI-MS (found 166.1386; calcd. 166.1358; found 168.1160; calcd 168.1150), established the molecular formula to be $C_{13}H_{22}O_3$ which was also confirmed by the presence of an ion at $m/z$ 244 ($M^+ + NH_4$) in the CI-MS spectrum.

Three oxygen atoms in the molecule were attributable to an $\alpha,\beta$-unsaturated carbonyl (1665 $cm^{-1}$) and an acetoxyl group (1730 $cm^{-1}$). Therefore, three unsaturations were due to these two carbonyls and the double bond.

By decoupled PMR experiments, five methyls were assigned to be a methyl (0.87) attached to a methylene, a methyl (1.02) attached to a methine, a methyl (1.09) attached to an $\alpha$-carbonylmethine, a methyl (1.79) attached to the double bond, and a methyl (2.08) in the acetoxyl group. These spectroscopic data indicated that compound C was a dehydro derivative of serricornin acetate. Judging from the presence of the conjugated double bond with carbonyl group in the molecule, 7-acetoxyl-4,6-dimethyl-4-nonen-3-one was assigned for the acetate of V.

*(2S,3R)-2,3-Dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (Serricorone, VI).* Compound VI was isolated from the 100% ether fraction and purified by preparative GC. MS (*m/z*): 238 ($M^+$, 15), 43(10),55(10), 57(77), 69(7), 83(18), 97(11), 109(15), 112(10), 113(31), 124(19), 139(7), 153(10), 182(100), 183(12); IR($cm^{-1}$, film); 2970(s), 2940(s), 2850(s), 1715(s), 1660(s), 1605(s), 1460(m), 1380(m), 1360(m), 1350(m), 1240(m), 1210(m), 1160(w), 1140(m), 1120(m), 1090(m), 1050(m), 960(m); PMR ($CDCl_3$, $\delta$ TMS, 400 MHz): 0.59, 0.60 (3H, t, $J$ = 7.3 Hz), 0.83, 0.85 (3H, d, $J$ = 7.3 Hz), 0.95, 0.97 (3H, t, $J$ = 7.3 Hz), 1.16, 1.18 (3H, d, $J$ = 6.8 Hz), 1.36 (2H, m), 1.768, 1.773 (3H, s), 2.10 (2H, m), 2.22 (1H, m), 3.11 (1H, q, $J$ = 6.8 Hz), 3.50 (1H, m); irradiation at 1.36, 2.10, 2.22, and 3.10 simplified the ts at 0.59, 0.60 to bds, the ts at 0.95, 0.97 to ss, the ds at 0.83, 0.85 to ss, and the ds at 1.16, 1.18 to ss, respectively; CMR ($CDCl_3$, $\delta$ TMS): 7.78, 9.5, 9.7, 12.7, 23.3, 23.6, 33.9, 42.7, 49.1, 49.5, 82.6, 83.3; signals of the carbons belonging to the carbonyls and the double bond were not observed.

Structural elucidation of serricorone (VI) by spectroscopic evidence and its synthesis has already been reported (Chuman et al., 1983).

*(2S,3R)-2,3-Dihydro-3,5-dimethyl-2-ethyl-6-(1-methyl-2-hydroxybutyl)-4H-pyran-4-one (Serricorole, VII).* Compound VII was isolated from the 100% ether fraction and purified by preparative GC. MS (*m/z*): 240($M^+$, 28), 57(10), 59(8), 70(8), 82(10), 83(35), 112(37), 113(100), 133(28), 145(11), 153(8), 182(43), 183(21); PMR ($CDCl_3$, $\delta$ TMS): 0.9–1.1 (12H, m), 1.4–1.8 (4H, m), 1.76(3H, s), 2.35 (1H, m), 2.88 (1H, m), 3.75 (1H, m), 4.06 (1H, m). Acetate of compound G was also obtained from the 100% ether fraction after acetylation. MS (*m/z*); 282 ($M^+$, 4), 43(83), 57(16), 69(18), 70(12), 83(52), 97(50), 101(23), 109(13), 113(16), 124(23), 125(15), 137(10), 152(25), 153(100), 171(29), 182(73), 193(51), 222(54); PMR ($CDCl_3$, $\delta$ TMS): 0.83–1.3 (12H, m), 1.4–1.8 (4H, m), 1.76 (3H, s), 1.97 (3H, s), 2.3–2.4 (1H, m), 2.95 (1H, m), 3.97 (1H, m), 5.02 (1H, m).

Structural elucidation of serricorole (VII) by spectroscopic evidence and its synthesis has already been reported (Chuman et al., 1983). Recently, the absolute configurations of $C_2$ and $C_3$ of serricorone (VI) and serricorole (VII) were established to be (2*S*,3*R*) by comparative study on the ORD curves of natural ones with that of stegobinone (XX), the sex pheromone of the drugstore beetle.[3]

*2,6-Diethyl-3,5-dimethyl-3,4-dihydro-2H-pyrane (Anhydroserricornin, II) and 4,6-Dimethylnonan-3,7-diol (IV).* Compound II and compound IV were detected in the 2–5% ether/hexane fraction. Mass spectra of compounds II and IV were identical to those of the synthetic anhydroserricornin (II) and 4,6-dimethylnonan-3,7-diol (IV), respectively.

[3]Private communication from Dr. Ebata and Prof. K. Mori, University of Tokyo, Japan.

*The Pheromone Activity of the Components*

The hexane extract of the female could elicit the pheromone activity, which was observed as sequential behavior of the males; an antennal elevation with mesothoracic legs, rapid zig-zag locomotion toward the pheromone source, and copulation attempts with other test males. To investigate the contribution of each component to the biology activity, the sex pheromone activity of each component was estimated by the behavioral bioassay and EAG experiments using synthetic materials. Three parameters were employed in our bioassay: (1) attractiveness—the number of males that climbed up the filter impregnated with test chemicals (total number of times males climbed up filter paper exceeds 10 because some males climbed up filter paper several times); (2) sex stimulation—the number of homosexual couples induced; and (3) reactivity—number of males responding among 10 test males (Figure 5).

Results of our bioassay indicated that ($S^*S^*S^*$)-serricornin[4] (I) could elicit the strongest sex pheromone activity on all parameters of this bioassay among the test chemicals. It showed that serricornin was the main component of the sex pheromone of this insect which could evoke strong attractiveness and sex stimulation for the males. Serricorone (VI) and serricorole (VII) could also stimulate strong sexual activity which induced the mass-mounting behavior among test males, but these components showed somewhat weak attractiveness. These components were considered to contribute to the supplementary factors of sex stimulation by cooperation with serricornin in the copulation of this insect. 4,6-Dimethylnonan-3,7-dione (III) and 4,6-dimethylnonan-3,7-diol (IV) had slight sex pheromone activity, but anhydroserricornin (II) elicited almost no typical activity. These results were entirely opposite to those reported by Levinson et al. (1981). Judging from the small amount of components II and IV in the female extract, these were not considered to play an important role in the occurrence of sex pheromone activity.

These observations were consistent with the results of EAG experiments except that of 4,6-dimethylnonan-3,7-dion III, the EAG intensity of which cannot be explained at present.

Consequently, it was demonstrated that sex pheromone activity of this insect was evoked mainly by serricornin, although it remained uncertain whether the active form was the acyclic keto alcohol or cyclic hemiacetal. Serricorone (VI) and serricorole (VII) might act with serricornin (I) as supplementary factors to enhance the sex stimulation in the copulation.

[4]In this bioassay ($S^*S^*S^*$)-serricornin, which was purified from stereoisomeric mixture of serricornin, was used as testing material. A recent study on the stereochemistry-pheromone activity of serricornin revealed that only ($S,S,S$)-serricornin, a naturally occurring isomer, had the strong sex pheromone activity among eight stereoisomers (Mochizuki et al., 1984).

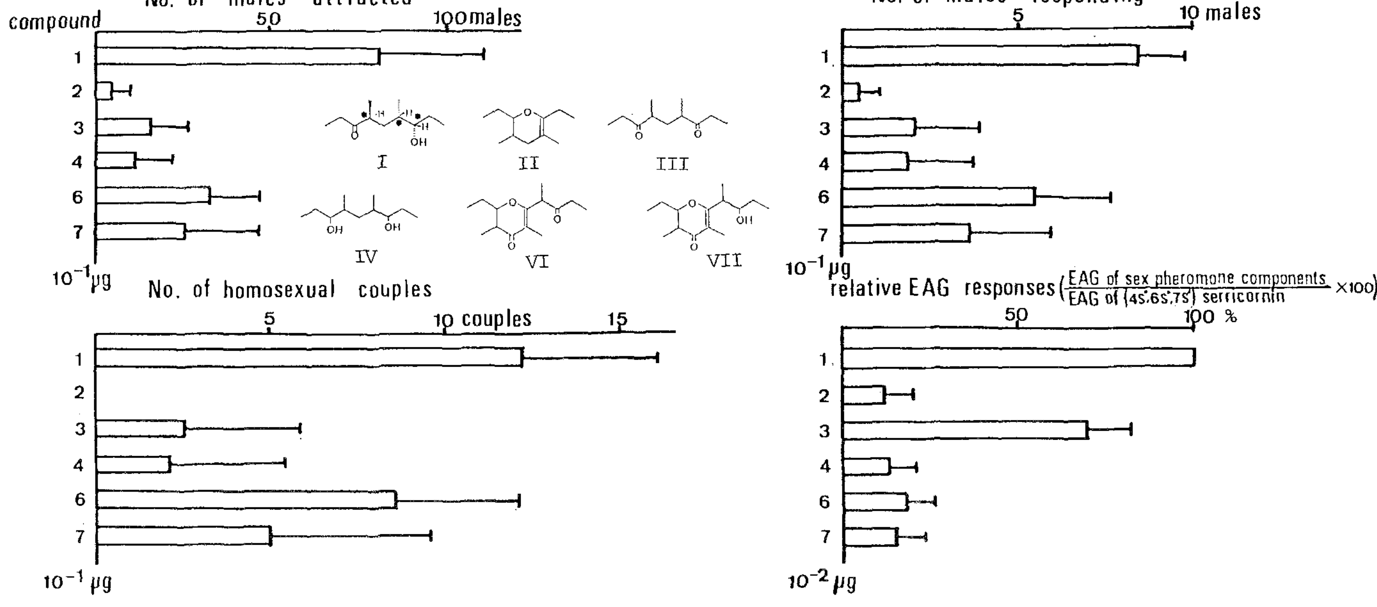


FIG. 5. Results of bioassay and EAG experiments with synthetic material. Ten unmated males were used in the bioassay with the observation time for 10 min. EAG responses of each component were normalized with the respect to those of ($S^*$,$S^*$,$S^*$)-serricornin.

### *Relationship between Sex Pheromone Components and Polyketide Biosyntheses*

Seven components isolated in our study on the sex pheromone of cigarette beetle could be divided into two groups according to the carbon skeletons of their gross structures. One of the groups was made up by C-11 series components, serricornin (I), anhydroserricornin (II), 4,6-dimethyl-nonan-3-one (III), 4,6-dimethylnonan-3,7-diol (IV), 4,6-dimethyl-7-hydroxy-4-nonen-3-one (V); and another was comprised of C-14 series components, serricorone (VI) and serricorole (VII). These components had structural similarities regarding the position of the functional groups and methyl branches in their structure. The 3,7-bifunctional and 4,6-dimethyl relationship in C-11 series could be also expanded to the 3,5,7,9 multifunctional and 4,6,8 trimethyl relationship in C-14 series. These structural features suggested that these C-11 and C-14 components might be derived from the corresponding C-11 and C-14 polyketide precursors which were formed by the condensation of four propionate units and five propionate units, respectively (Chuman, 1981b) (Figure 6).

It could be assumed that C-11 precursor 3,5-dioxo-7-ol (XXIV), was converted to 3-oxo-5,7-diol by regioselective hydrogenation of $C_3$-carbonyl group, followed by dehydration to give 4,6-dimethyl-7-hydroxy-4-nonen-3-one (V). Stereoselective hydrogenation of the double bond in V furnished serricornin (I),

FIG. 6. Possible polyketide biosynthesis of the sex pheromone components of the cigarette beetle.

which formed the equilibrium between the acyclic keto alcohol and cyclic hemiacetal structures.

This equilibrium of serricornin (I) gave anhydroserricornin (II) by dehydration, 4,6-dimethyl-nonan-3,7-dione (III) by oxidation of the $C_7$-hydroxyl, and 4,6-dimethyl-nonan-3,7-diol (IV) by hydrogenation of the $C_3$ carbonyl. It was also shown that the C-14 precursor, 3,5,7-trioxo-9-ol (XXVII), might furnish serricorone (VI) by dehydrative cyclization between $C_5$ and $C_9$, followed by the selective hydrogenation of the carbonyl in the side chain to give serricorole 7. Another route for the formation of serricorone (VI) from $C_{11}$ pyranone by an aldol condensation with a propionate unit could be considered.

This assumption of polyketide biosynthesis was supported by the biomimetic syntheses of serricorone (VI) and stegobinone (XX), which were synthesized via polyketide precursors (Ansell et al., 1979; Sakakibara and Mori, 1979; Chuman et al., 1983; Ono et al., 1983).

Many biologically active substances of insects that seemed to be related to the polyketide biosynthesis were reported as defensive substances of opilionids, (VIII-XII, XV, XXIII) (Jones et al., 1976, 1977), and of mutilid wasps, (XII, XVI, XVII) (Fales et al., 1980), alarm pheromone of ants (XII-XIV) (Fales et al., 1972; Riley et al., 1974), an aggregation pheromone of bark beetle (XII,

FIG. 7. Biologically active substances of insects related to polyketide biosynthesis.

XIX) (Pearce et al., 1975), an aggregation pheromone of rice and maize weevil (XXII) (Schmuff et al., 1984), and a sex pheromone of drugstore beetle (XX) (Kuwahara et al., 1978) (Figure 7).

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# INFLUENCE OF CAGE DESIGN ON PRECISION OF TUBE-TRAP BIOASSAY FOR ATTRACTANTS OF THE ONION FLY, *Delia antiqua*[1,2]

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**Abstract**—Responses of onion flies, *Delia antiqua,* to known attractants were measured in the laboratory with a novel tube-trap bioassay. The relative numbers of flies caught in tube traps baited with enzymatic yeast hydrolysate, brewer's yeast, and *n*-dipropyl disulfide were similar to those obtained previously with cone traps in the field. Changing the shape of the bioassay cage from a cuboid to a cylinder decreased the experimental error obtained from analysis of variance, as did rotating the floor of the circular cage. This bioassay should be useful in evaluating attractants for other insects that orient along the substrate.

**Key Words**—*Delia (Hylemya) antiqua,* Diptera, Anthomyiidae, onion fly, onion maggot, tube trap, insect attractants.

## INTRODUCTION

For more than a decade, vegetable entomologists have been interested in developing attractants for monitoring the onion fly, [*Delia antiqua* (Meigen)], or for use in poisoned baits. Since decomposing onions have been found to be one of the most potent attractants of onion flies (Dindonis and Miller, 1980a; Ishikawa et al., 1981), we sought to generate decomposing onions and extract the attractants therefrom. Since not all rots developed by onion tissue are equally attractive to onion flies (Miller et al., 1984), it became necessary to assay rotting

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onions for attractancy prior to extraction to ensure that the starting material was indeed attractive. Assaying attractancy in the field had several drawbacks, however.

First, the attractancy of microbe-infested onion tissue changes dramatically over time (Miller et al., 1984). By the time sufficient flies were caught in cone traps (Dindonis and Miller, 1980a) to permit statistical analysis of results, the once attractive material was often beyond its prime. Complicating this problem are fluctuations in fly activity in the field due to meteorological conditions and fluctuations in fly populations due to natural phenology. Here we report a rapid laboratory bioassay for *D. antiqua* attractants as well as modifications that can maximize its precision.

## METHODS AND MATERIALS

The bioassay traps (tube traps, Figure 1), were 400-ml glass beakers with three equally spaced holes (1 cm diam) around the basal circumference. The holes were placed at the bottom of the traps since earlier work (Dindonis and Miller, 1980b) showed that onion flies approach sources of volatiles via short, hopping flights along the substrate. Inserted through the holes were 4-cm lengths of glass tubing (0.8 cm ID), which projected ca. 1 cm outside the beaker. The entrance tubes decreased the random entry of flies into the beaker. Sitting on

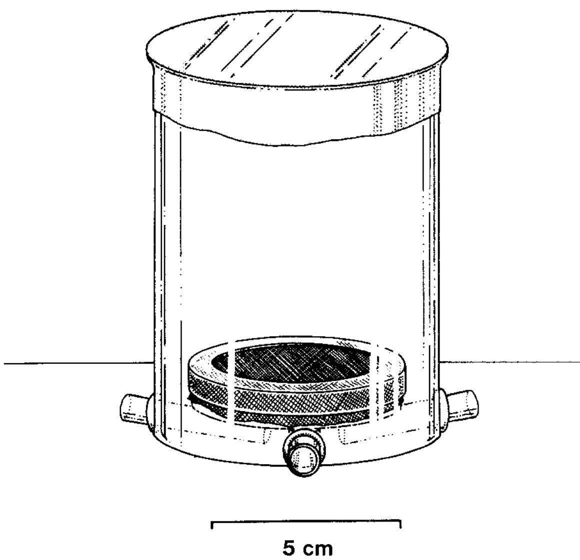


FIG. 1. Tube trap for assaying onion fly attractants.

top of these tubes was a 5.5-cm Petri dish containing the test material covered with plastic screening to prevent contact of the flies with the bait. Assembly of a tube trap was completed by covering the beaker with Parafilm.

Three cage designs were compared in this study. The first was a cuboid (80 × 60 × 60 cm) with a screen ceiling and plastic sides. The bottom 10 cm of the back and side walls were screen, and the floor was asbestos board. The other two cages were acetate cylinders (55 cm diam × 60 cm) with Plexiglas lids. In the center of each lid was a 10-cm hole covered with plastic screen for ventilation. One of these cages (designated "circular cage") had a stationary floor of screen and hardware cloth, while the other (designated "rotating cage") rested over a screen disk that rotated at 4 rph when powered by a small electric motor. The gap between the disk and the sides of the cylinder was blocked with a length of foam weatherstripping. Each cage had a small (ca. 200 $cm^2$) plastic door that permitted access to the inside.

The cages were placed in a controlled-environment chamber maintained at 21 ± 1°C and 35 ± 5% relative humidity under a 16:8 light–dark regime. Flies were provided with water continuously and the diet of Ticheler (1971) between replicates. Food was removed during bioassays to increase the responsiveness of the flies to baits. The flies were drawn from a population that had been in laboratory culture for two years. To ensure uniformity of age structure, only those flies eclosing during a 4-day span were included in a common stock cage. Experimental cages were stocked by first aspirating 600 flies of each sex, in groups of 10, from the stock cage. Groups were then chosen at random and assigned to the experimental cages in rotation, until each cage contained 200 flies of each sex. As flies died during the experiment, they were removed and replaced with new ones from the stock cage.

We elected to test only four baits simultaneously since the traps could be spaced uniformly in the rectangular cage (i.e., one in each corner). Treatments chosen covered a range of attractancy to adult females, based on trap catch data from the field (Miller and Haarer, 1981). Enzymatic yeast hydrolysate (EYH; ICN, Cleveland, Ohio) was the most attractive treatment, while brewer's yeast (BY; Bio-Serv, Frenchtown, New Jersey) and *n*-dipropyl disulfide ($Pr_2S_2$; Eastman Kodak, Rochester, New York) were intermediate; an empty trap served as a negative control. The yeast baits were presented as 5 g powder, while $Pr_2S_2$ was presented as 100 $\mu$l of 0.7 mole fraction in peanut oil in a size 3 BEEM polyethylene enclosure (Dindonis and Miller, 1981).

The experimental design was randomized complete block, with a total of six blocks conducted (over time) per cage. The three cages were tested simultaneously, with 1–3 days between blocks. Traps were placed at the corners of an imaginary square, 45 cm on a side, centered in each cage. Treatments were assigned to positions at random for each block. After 24 hr, flies caught in the traps were sexed, counted, and released back into their respective cages. Data

for each cage were analyzed separately with analysis of variance (ANOVA), and the mean square errors (MSEs) from the analysis of each cage were compared using Bartlett's test for homogeneity of variances (Steel and Torrie, 1980).

## RESULTS

The numbers of male flies caught were very low, averaging less than 15% of the total flies caught. Therefore, only numbers of females were analyzed. The relative numbers of females caught by the treatments (Table 1) were similar to field-trapping results, except that EYH and BY caught nearly the same numbers of flies in the laboratory, while EYH caught several-fold more flies than BY in the field (Miller and Haarer, 1981). However, the same trends in trap catch were observed in all three cages, indicating uniformity of response of the

TABLE 1. FEMALE ONION FLY CATCHES IN TUBE TRAPS AS AFFECTED BY CAGE DESIGN

| | Mean trap catch[a] (±SD) | | |
|---|---|---|---|
| Treatment | Rectangular | Circular | Rotating |
| EYH | 18.7 ± 11.7a | 12.8 ± 4.6a | 16.8 ± 6.3a |
| BY | 11.0 ± 11.4ab | 14.8 ± 3.5a | 15.1 ± 6.3ab |
| $Pr_2S_2$ | 7.0 ± 8.9bc | 10.7 ± 6.5a | 9.8 ± 6.0b |
| Control | 0.8 ± 0.8c | 2.0 ± 1.3b | 2.2 ± 1.2c |
| Total | 37.5 ± 20.9 | 40.3 ± 7.7 | 43.9 ± 13.2 |

[a] Means followed by the same letters within columns are not significantly different at the 5% level as determined by the LSD test on data transformed to $(x + 0.5)^{1/2}$.

TABLE 2. COMPARISON OF MSEs AND TREATMENT $F$ VALUES FROM SEPARATE ANOVAs OF TRAP CATCHES (SQUARE ROOT-TRANSFORMED) IN EXPERIMENTAL CAGES

| Cage type | MSE[a] | Treatment $F$[b] |
|---|---|---|
| Rectangular | 1.76a | 5.69** |
| Circular | 0.71ab | 9.47*** |
| Rotating | 0.48b | 16.25*** |

[a] Mean square errors followed by the same letter are not statistically different at the 5% level as determined by pairwise Bartlett's tests for homogeneity of variances with 15 degrees of freedom.

[b] **, significant at the 0.01 level; ***, significant at the 0.001 level.

three fly populations and similar performance of traps in each cage type. The total numbers of flies caught per cage per replicate were not statistically different ($F_{2,10}$ = 0.54, square root-transformed data).

A measurement of the ability to detect treatment differences is the MSE from analysis of variance. A comparison of the MSEs and treatment $F$ values among cage types (Table 2) shows that the rectangular cage had the highest MSE and lowest treatment $F$, while the rotating cage had the lowest MSE and the highest treatment $F$. In addition, the rotating cage had the highest degree of homogeneity of treatment variances according to Bartlett's test ($\chi^2 = 4.76$, ns), while the rectangular cage had the lowest ($\chi^2 = 9.39$, $0.01 < P < 0.05$).

## DISCUSSION

A desirable feature of any bioassay is that it be able to detect differences between treatments as quickly as possible. Since responses of caged *D. antiqua* to attractants have large variances associated with them, it is often necessary to replicate such bioassays many times. Increasing the precision (i.e., the ability to detect treatment differences by decreasing experimental error) of the bioassay decreases the number of replicates needed to detect differences at the same confidence level and can therefore facilitate the isolation of biologically active materials.

Modifying the shape of the bioassay cage from a cuboid to a cylinder was prompted by the preliminary observation that traps in some positions in the rectangular cage caught more flies than others regardless of the treatments placed there. This modification resulted in a large decrease in the experimental error and a corresponding increase in the treatment $F$ value for the same number of treatments and replicates in each cage (Table 2). This improvement was likely due to the removal of corners from the cage. If flies favor certain corners of the cage more than others, this would increase their chances of randomly entering a trap in that location. Obviously, a circular cage has no corners and is therefore less susceptible to such effects. Refining the circular cage by rotating the floor further improved the precision of this bioassay as judged by the decrease in MSE and the increase in treatment $F$ value. This increased precision can most likely be attributed to allowing each treatment to spend equal time in all positions within the cage. This same result can be achieved by manually rotating treatments on a regular schedule, but having the floor rotate by itself reduces labor and allows the assay to run unattended for considerable lengths of time. Although the percentage decrease in MSE of the circular vs. the rotating circular cage was smaller than the rectangular vs. circular cage (32% as opposed to 60%), the additional gain in precision may be justified since the best separation of treatment means was obtained in the rotating cage.

The rotation of treatments to reduce experimental error is not a new idea.

For example, DeVaney et al. (1971) used a rotating cage when measuring the response of screwworm flies to potential attractants, and Ellis and Hardman (1975) placed test plants on turntables inside their bioassay cages when measuring the responses of cabbage root flies. The intuitive advantage of using rotation to even out exposure of treatments to locations in the cage is substantiated by the significant decrease in MSE in the experiments reported here.

We believe the tube traps used in these experiments measure attraction, albeit indirectly. Since the flies must pass through narrow tubes to enter the trap, it is unlikely that arrestment is the mechanism responsible for flies accumulating in the trap. Rather, it is much more likely that some chemotactic mechanism is responsible for guiding the flies into a trap, and thus the traps can be said to measure attractancy of the test materials.

This laboratory bioassay should prove to be useful for measuring the attractancy of test materials to a variety of other insects. Such insects might include other anthomyiid flies, beetles, and most other insects that approach the source of an attractant along the substrate.

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# ISOLATION OF *C*-GLYCOSYLFLAVONES AS PROBING STIMULANT OF PLANTHOPPERS IN RICE PLANT

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**Abstract**—Three species of planthopper, *Nilaparvata lugens*, *Sogatella furcifera*, and *Laodelphax striatellus*, showed characteristic behavior of stylet probing in parenchymal tissues of plants. Feeding experiments of planthoppers on aqueous sucrose solution containing the extract of rice plants or barnyard grass revealed the presence of the stimulant for the probing behavior in the plant tissues. Eight *C*-glycosylflavones which stimulated stylet probing were isolated from rice plants. Four of them were identified to be schaftoside, neoschaftoside, carlinoside, and neocarlinoside, the last one being a new compound isolated from the plant. The isolated *C*-glycosylflavones showed the same level of the activity for the probing of *N. lugens* as the rice plant extract only when all of them were combined.

**Key Words**—*Nilaparvata lugens*, *Sogatella furcifera*, *Laodelphax striatellus*, Hemiptera, Delphacidae, kairomone, probing stimulant, *C*-glycosylflavone, schaftoside, neoschaftoside, carlinoside, neocarlinoside.

## INTRODUCTION

Three species of planthopper, the brown planthopper, *Nilaparvata lugens* (Stål), the white-back planthopper, *Sogatella furcifera* (Horváth), and the smaller brown planthopper, *Laodelphax striatellus* (Fallén), are important pests of rice in Asian countries, causing significant loss of the yield either by sucking plant sap or by transmitting virus diseases. *N. lugens* has recently become a major pest of rice plant in several countries of tropical Asia (Dyck and Thomas, 1979).

The feeding process of planthoppers can be divided into two distinct behavioral phases (Sōgawa, 1982): probing, which is performed in indigestible parenchymal tissues, and sucking, which is done after stylet insertion into di-



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gestible vascular bundles. When planthoppers feed on plants, they first secrete a small amount of saliva on the plant epidermis, which immediately coagulates to form a "feeding mark" (Naito, 1964), and then insert their stylet through it into the plant tissues. During stylet insertion, planthoppers eject coagulable saliva, which sets rapidly to a gel encasing the protruded stylet forming a stylet sheath that is left in the plant tissues even after withdrawal of the stylet. Planthoppers repeat stylet probing to leave branched stylet sheaths originating from a "feeding mark" until the stylet encounters the vascular bundles from which they suck phloem sap. The sucking behavior is suppressed during the probing behavior. The stimuli involved in each behavioral response are designated the probing stimulant and sucking stimulant, respectively. These two behavioral responses are independent and incompatible and are under the control of particular sets of stimuli occurring in either parenchymal tissues or vascular bundles respectively (Sōgawa, 1974).

Various known plant secondary substances and their related compounds have been assayed, and salicyclic acid and four flavonoids were reported as the probing stimulants for *N. lugens* in rice plant (Sōgawa, 1974, 1976). Aside from planthoppers, the probing behavior of some aphids is affected by sinigrin (Nault and Styer, 1972), phlorizin (Klingauf, 1971), and *n*-paraffin (Klingauf et al., 1971). These studies demonstrated the presence of probing stimulants; the compounds subjected to the assay, however, were known plant secondary substances which had been isolated for another purpose. Little effort has been made to isolate probing stimuli from the host plant.

The present paper deals with isolation of *C*-glycosylflavones from rice plant as kairomones stimulating probing behavior of planthoppers and also presents the response of *N. lugens* to the isolated compounds.

## METHODS AND MATERIALS

*Insect.* Macropterous females of three species of planthoppers (*N. lugens*, *S. furcifera*, and *L. striatellus*) were taken from their stock colonies which were reared successively on rice seedlings at 25°C, 14 hr illumination.

*Plant.* Rice plants (cv. Nihonbare) and barnyard grass (*Echinochloa crus-galli* var. *oryzicola*) were grown in a green house. Rice plants for extraction of the probing stimulant were collected in a paddy field that had not been treated with pesticide.

*Bioassay for Probing Response.* Planthoppers produce stylet sheaths, similar to those produced in rice plant tissues, when they are reared on an aqueous solution of sucrose containing the plant extract under a thin film of polyethylene (Figure 1) (Sōgawa, 1974). Since stylet sheaths deposited on the film are clearly observable with a microscope after dyeing with a basic fuchsin solution, it is

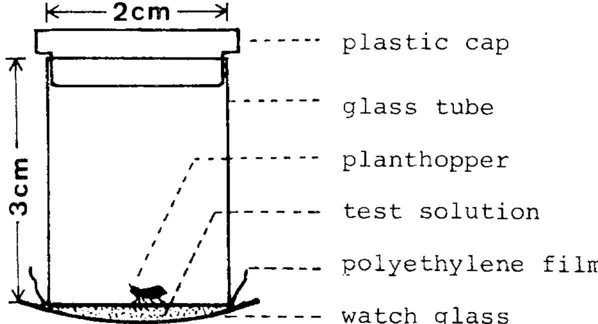



FIG. 1. Bioassay apparatus for probing response of planthopper to various test media.

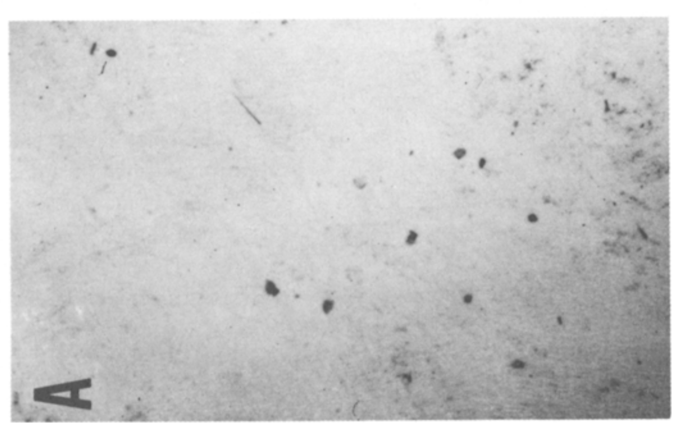
possible to evaluate the probing activity by counting the number of stylet sheaths formed on the film.

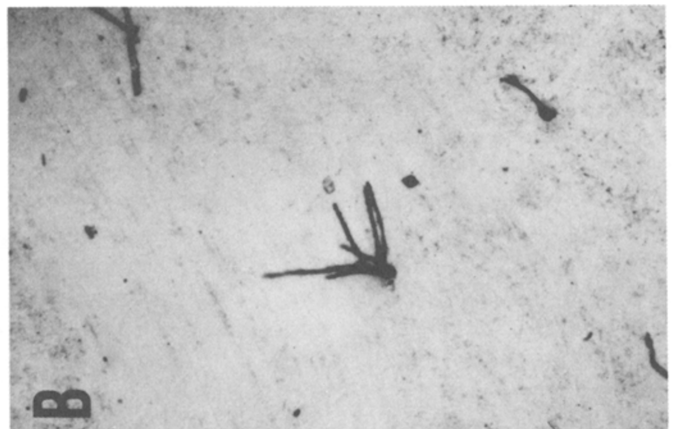
The assay used in this study consisted of 10 planthoppers in the apparatus illustrated in Figure 1 and kept in a moistened box for 24 hr, at 25–28°C and 85–95% relative humidity, and under continuous illumination. At the end of the test period, stylet sheaths deposited on the film were washed with water, stained with a basic fuchsin solution, and observed with a microscope. In order to compare the probing activity quantitatively, elongated sheaths (longer than 0.1 mm) were classified according to their forking pattern into the following four groups: nonbranched, two-branched, three-branched, and more than four-branched, to each of which was given the coefficient 1, 2, 3, and 4, respectively. The activity was expressed by a response value that was the summation of the number of the observed sheaths multiplied by their coefficient. All tests were replicated five times.

## RESULTS AND DISCUSSION

*Probing Response to Sucrose and Plant Extracts.* The plant extract was obtained as follows: Fresh leaves and stems of rice plant (400 g) were cut in rather large pieces (about 10 cm in length), immersed in methanol (600 ml) for 4 days, decanted, and this procedure was repeated three times. The combined methanol solution was evaporated under reduced pressure. The residue was dissolved in water (300 ml) and washed successively with hexane, chloroform, and ethyl acetate (100 ml × 3, each). The aqueous layer was evaporated to dryness to give a brown oil (10.5 g, 2.6% yield from fresh rice plant), which is refered to as "the rice plant extract." Barnyard grass was extracted by the same procedure.

As shown in Figure 2, stylet sheaths were produced by *N. lugens* on a



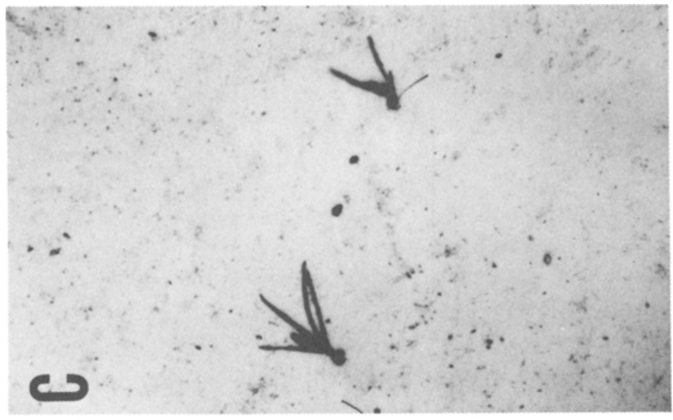




FIG. 2. The stylet sheaths produced in 15% sucrose alone (A), containing 2% the rice plant extract (B), 2% the barnyard grass extract (C).

polyethylene film in the probing response test with 15% sucrose solution containing either the rice plant extract or the barnyard grass extract at the concentration of 2%. *N. lugens* deposited many long and branched stylet sheaths in both the rice plant and barnyard grass extracts as the insect does in plant tissues. On the other hand, in the solution of sucrose alone *N. lugens* deposited only a few short sheaths, leaving many "feeding marks" on the external surface of the film at the points of the stylet penetration. The activities expressed by the response value, shown in Table 1, indicate that the stimulant for probing of *N. lugens* is contained in both the rice plant and barnyard grass extracts.

The probing behavior of two other species of planthopper, *S. furcifera*, and *L. striatellus*, is stimulated by the rice plant extract to the same extent as *N. lugens* (Table 2).

As reported previously (Kim et al., 1975, 1976), the rice plant is a host plant to *N. lugens*, while barnyard grass is not because it contains a potent sucking inhibitor, *trans*-aconitic acid, which is found in many kinds of plants (Stout et al., 1967). Based on results of these experiments there is little doubt that the probing and sucking behavior of *N. lugens* are controlled by different stimuli; probing is stimulated by the extract of rice plant and also of barnyard grass but not by sucrose, while sucking is activated by rice plant extract and also by sucrose but inhibited by the barnyard grass extract.

*Preliminary Separation of Probing Stimulant.* Prior to isolation of the probing stimulant from rice plant, the chemical nature of the stimulant was explored on a small scale. The rice plant extract (1 g) was separated into neutral (579 mg), acidic (104 mg), and basic (120 mg) fractions by column chromatography on a cation-exchange resin (Dowex 50W × 8, $H^+$-form) eluted with 2 N $NH_4OH$ and subsequently on an anion-exchange resin (Dowex 1 × 8, formate form) eluted with 20 N formic acid. Only the acidic fraction showed activity for

TABLE 1. PROBING RESPONSES OF MACROPTEROUS FEMALES OF *N. lugens*

| | 15% sucrose | | 2% rice plant extract plus 15% sucrose | | 2% barnyard grass extract plus 15% sucrose | |
|---|---|---|---|---|---|---|
| No. of branches | Observed no. | Multiplied no. | Observed no. | Multiplied no. | Observed no. | Multiplied no. |
| 1 | 2 | 2 | 29 | 29 | 26 | 26 |
| 2 | 1 | 2 | 11 | 22 | 16 | 32 |
| 3 | 0 | 0 | 11 | 33 | 19 | 57 |
| ≥4 | 0 | 0 | 38 | 152 | 30 | 120 |
| Response value[a] | | 4 | | 236 | | 235 |

[a]Response value was calculated according to the method described in the text.

TABLE 2. PROBING RESPONSES OF THREE SPECIES OF PLANTHOPPER

| | Mean response value[a] | | |
|---|---|---|---|
| Component in 15% sucrose | *N. lugens* | *S. furcifera* | *L. striatellus* |
| None | 3.2a | 3.0a | 3.2a |
| 2% rice plant extract | 229.4b | 241.6b | 237.0b |
| 2% barnyard grass extract | 234.0b | | |

[a] Mean based upon five replicates. Response value of each test ($X$) was transformed to $\sqrt{X + 0.5}$ for analysis. Mean followed by different letters within a column are significantly different (Duncan's multiple-range test, $P < 0.01$).

probing stimulation at a concentration equivalent to 2% solution of the original extract. The acidic fraction was eluted from a column of anion-exchange resin with stepwise increasing concentration of formic acid in five fractions. Bioassay showed that the 3 N formic acid eluate was significantly more active than the other fractions.

The 3 N formic acid eluate was further purified by chromatography on a column of silicic acid (Kieselgel 60, 25 g) eluted successively with ether, ether–ethyl acetate (1:1), ethyl acetate, ethyl acetate–methanol (1:1), and methanol. On bioassay, the same level of the activity as the rice plant extract was found in the ethyl acetate–methanol eluate. Addition of ether to this eluate resulted in precipitation of an active yellowish brown powder.

Paper chromatography (Whatman 3MM, developed with the upper layer of the mixture of *n*-butanol, acetic acid, and water (4: 1:5) suggested that the active powder contained at least three components, A, B, and C, their $R_f$ values being 0.60, 0.43–0.48, and 0.38, respectively. These spots on the paper were detectable visually by a pale yellow color without any treatment and by a more intense yellow or orange color with a spray of 1% sodium carbonate solution. With 1% aluminum chloride solution, they gave a yellow or orange color, and on illumination of long-wave UV light displayed a yellow fluorescence. With 2% zirconium oxychloride in methanol they showed a yellow or orange color which disappeared after a spray of a 5% citric acid solution. In the UV absorption spectrum, the active powder gave two absorption maxima at around 270 nm and 320–350 nm. These chromatographic behaviors and color reactions as well as the UV spectra suggested that the active powder was a mixture of flavonoid compounds (Markham, 1975; Markham and Mabry, 1975).

*Isolation of Probing Stimulant.* Purification procedures of the probing stimulant in rice plant are summarized in Figure 3. A methanol extract of rice plant (5 kg, fresh weight) was dissolved in water (3 liters) and washed with hexane, chloroform, and ethyl acetate successively (each three times with a 1-

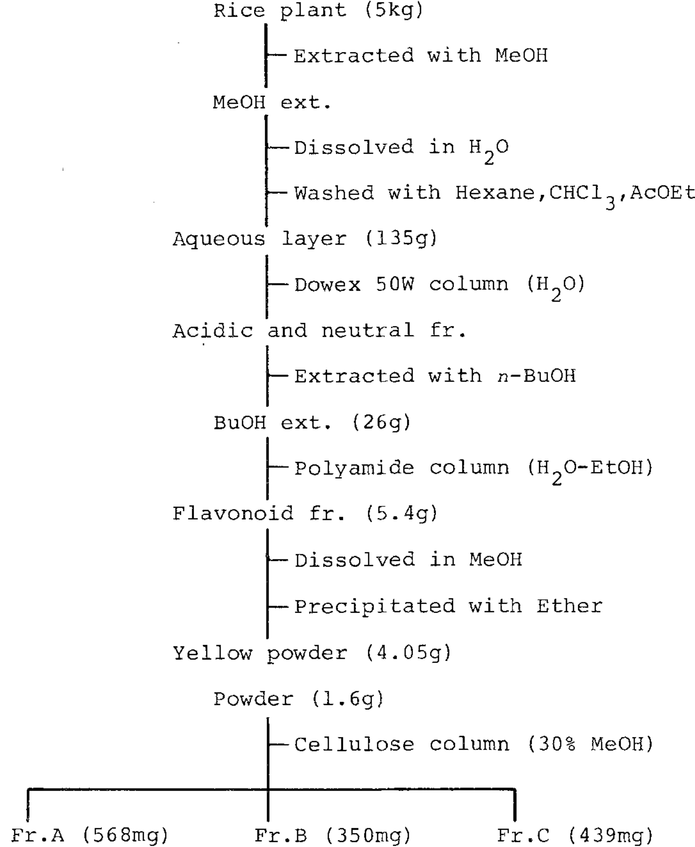



FIG. 3. Isolation procedure of the probing stimulant in rice plant.

liter portion) to separate inactive substances. Evaporation of the aqueous solution gave an active oil (135 g). The oil was passed through a column of cation-exchange resin to give a fraction containing neutral and acidic compounds, which was repeatedly extracted with *n*-butanol. The butanol extract (26 g) was divided into three portions, each of which was chromatographed on a column of polyamide (3.6 × 56 cm) eluted with water; 30%, 50%, and 70% aqueous ethanol; and methanol successively. The 30% and 50% ethanol eluates containing visibly yellow colored bands were combined and evaporated to dryness to give a yellow oily residue, to which ether was added to obtain, on filtration, a yellow powdery precipitate (4.05 g in total).

A portion (200 mg) of the yellow powder was further chromatographed on a cellulose column (5 × 50 cm) eluted with 30% aqueous methanol. Each eluate (about 15 ml) was monitored by UV absorption at 340 nm and divided into three

fractions corresponding to fractions A, B, and C described above and shown in Figure 4. Repeating the same chromatographic procedure with eight portions of the yellow powder (8 × 200 mg = 1.6 g) yielded, on evaporation, three active powders, A (568 mg), B (350 mg), and C (439 mg), each of which still appeared to be composed of two or more compounds by paper chromatography.

It was difficult to separate fraction A into its components, while fractions B and C were fractionated, without any difficulty, each into two compounds by chromatography on a column of cellulose eluted with water-saturated *n*-butanol; $B_1$ (23 mg), and $B_2$ (13 mg) were given from B (38 mg), and $C_1$ (35 mg) and $C_2$ (20 mg) from C (80 mg).

Paper chromatography of fraction A indicated that it contained four compounds, $A_1$–$A_4$. Fraction A (164 mg) was fractionated in a dark room on a column of polyamide (2.7 × 42 cm) eluted successively with water and 20%, 30%, 40%, and 50% aqueous methanol (400 ml each). In the course of the chromatography the progress of the yellow bands on the column was monitored by UV light, and each eluate (10 ml) was also analyzed on a paper chromatogram developed with water-saturated *n*-butanol, and as a result, fraction A could be separated into three fractions, $A_1$, $A_2$ and $A_3$, and $A_4$.

$A_1$ was further purified on a column of cellulose eluted with water-saturated *n*-butanol to isolate compound $A_1$ (14 mg). The mixture of $A_2$ and $A_3$ was first subjected to cellulose column chromatography eluted with 20% aqueous methanol to be separated into three fractions, the first one rich in $A_2$, the second one being a mixture of $A_2$ and $A_3$, and the last one rich in $A_3$. The first and last one were separately rechromatographed on cellulose columns eluted with water-

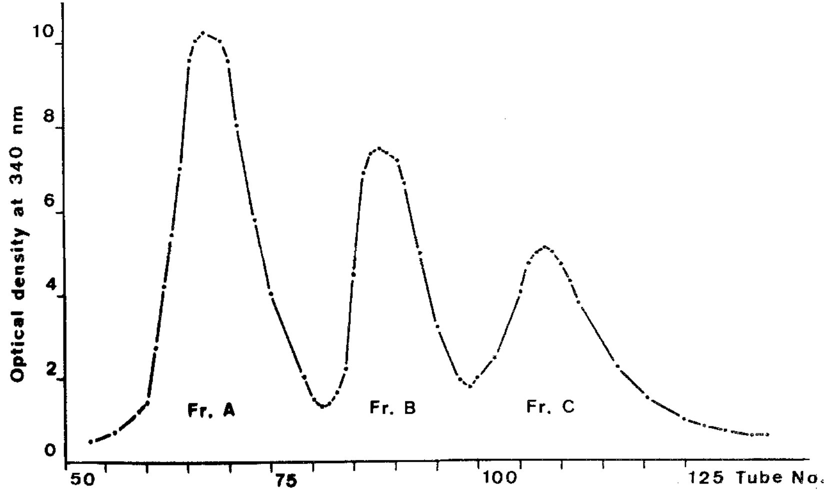



FIG. 4. Cellulose column chromatogram of the probing stimulant.

Glc=β-D-glucopyranosyl

X=β-L-arabinopyranosyl

$B_1$:$R_1$=H, $R_2$=OH (neoschaftoside)

$C_1$:$R_1$=$R_2$=OH (neocarlinoside)

X=α-L-arabinopyranosyl

$B_2$:$R_1$=H, $R_2$=OH (schaftoside)

$C_2$:$R_1$=$R_2$=OH (carlinoside)

FIG. 5. Structures of $B_1$, $B_2$, $C_1$, and $C_2$.

saturated *n*-butanol to give compound $A_2$ (22 mg) and $A_3$ (10 mg), respectively. The $A_4$ fraction was subjected to successive chromatography, first on a cellulose column eluted with *n*-butanol–acetic acid–water (4 :1 :1) and then on a column of Sephadex LH-20 eluted with methanol to isolate pure compound $A_4$ (28 mg).

*Structure Elucidation of Isolated Compounds.* Results of preliminary UV, NMR, and MS, and acidic hydrolysis studies indicated all eight isolated compounds to be *C*-glycosylflavonoids. Mass spectrometry of the permethylated derivatives of the compound and direct comparison on thin-layer chromatograms with authentic samples[1] show that $A_1$–$A_4$ are derivatives of 2″-*O*-hexosyl-6-*C*-hexosylluteolin; $B_1$ and $B_2$ are neoschaftoside and schaftoside, respectively; $C_1$ is neocarlinsoide (Linard et al., 1982), a new naturally occurring di-*C*-glycosylflavone; and $C_2$ is carlinoside (Figure 5, where $A_1$–$A_4$ are excluded because the structure for each compound remains to be confirmed).

*Probing Response to Isolated C-Glycosylflavones.* Eight *C*-glycosylflavones were isolated from rice plant as the stimulant for probing behavior of *N. lugens* as described above. Planthoppers may show probing behavior only in indigestible parenchymal tissues in the plant. It is not possible to estimate the exact content of these *C*-glycosylflavones in parenchyma. Based on the isolation yield of the active yellow powder from fresh rice plant (4 g from 5 kg of fresh rice plant as shown in Figure 3), however, it seems reasonable that the *C*-gly-

[1] These studies on the structure elucidation were made in cooperation with Prof. J. Chopin, Université Claude Bernard Lyon I, and will be reported in the near future (Besson et al., 1985).

cosylflavones in total are contained in rice plant at more than 800 ppm, taking into account some loss in the yield during the course of the isolation procedure. Consequently bioassays for estimation of the probing stimulatory effect were made in 15% aqueous sucrose containing the test material at the concentration of 1000 ppm and, if necessary, at 500 ppm or 2000 ppm.

As mentioned already, the rice plant extract stimulates the probing behavior of *N. lugens* at the concentration of 2%. The combined solution of an equal amount of fractions A, B, and C shows the same level of activity as the rice plant extract at 1000 ppm, while each fraction alone is much less active, as shown in Table 3. This indicates that the stimulatory activity for probing is not attributable to a single component but to several components combined. The combined solution of the isolated *C*-glycosylflavones is as active as the rice plant extract at 1000 ppm in total in which each of them is contained in the proportion shown in Table 3, indicating that the probing stimulant of planthoppers consists of eight *C*-glycosylflavones. Although the activity of each *C*-glycosylflavone alone was not assayed, owing to the small amount of available sample, it can be assumed that each of them shows much less activity as in the case of fractions A, B, and C.

In a series of studies on the feeding behavior of *N. lugens*, Sōgawa (1974, 1976) pointed out that salicylic acid and four flavonoids of rice plant (tricin-5-glucoside, glucotricin, orizatin, and homoinetin) were active as the probing stimulant for *N. lugens* when various known plant secondary substances and their related compounds were subjected to the assay. It was also reported that each solution of orizatin and homoinetin stimulated probing at concentrations of 100–1000 ppm. The structural elucidation of these two flavenoids has not been established (Kuwatsuka, 1962). Comparison of their UV spectra and $R_f$ values

TABLE 3. PROBING RESPONSES TO ISOLATED COMPOUNDS FROM RICE PLANT[a]

| | 500 ppm | 1000 ppm | 2000 ppm |
|---|---|---|---|
| Fr A + Fr B + Fr C (1:1:1) | 128.2 | 221.0a | 196.6 |
| Fr A | | 85.4d | |
| Fr B | | 75.4d | |
| Fr C | | 105.0c | |
| $(A_1 + A_2 + A_3 + A_4) + (B_1 + B_2) + (C_1 + C_2)$ (1:1:1:1) + (1.5:1.5) + (1.5:1.5) | 87.2 | 152.0b | 261.0 |

[a]Values represent the mean response values. Mean based upon five replicates. Response value of each test ($X$) was transformed $\sqrt{X + 0.5}$ for analysis. Means followed by different letters within a column are significantly different (Duncan's multiple-range test, $P < 0.01$).

on paper chromatogram with those of our samples indicates that orizatin and homoinetin may be di-*C*-glycosylflavones, corresponding to $B_2$ (schaftoside) and $C_1$ (neocarlinoside), respectively.

Some discrepancy is found in the concentration of the flavones necessary to elicit the stimulatory effect on the probing of *N. lugens* between assay results of Sōgawa's and ours. It may be concluded, however, that eight *C*-glycosylflavones were isolated from rice plant as the probing stimulant of planthoppers and showed the same level of activity as the rice plant extract only when they were combined.

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# ROLE OF HOST-PRODUCED STIMULI AND LEARNING IN HOST SELECTION BEHAVIOR OF *Cotesia* (=*Apanteles*) *marginiventris* (CRESSON)[1-3]

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**Abstract**—The study was conducted to determine how various factors, including learning, influence the host-selection behavior of the larval parasitoid *Cotesia* (=*Apanteles*) *marginiventris* (Cresson). Frass, silk, and feeding damage from *Spodoptera frugiperda* (J.E. Smith) larvae elicited visits and antennal examinations by females of the parasitoid. Learning played a role in the effective response to these stimuli in that previous contact experience with hosts and/or chewed leaves, exuviae, frass, or hemolymph significantly enhanced the response of the parasitoid. Previous contact with host plants alone did not improve the responses. Experienced parasitoids were more active and spent less time at rest than inexperienced parasitoids. Previous experience was also found to be of significant importance in the ability of the parasitoid to discriminate between parasitized and unparasitized host larvae.

**Key Words**—Kairomones, learning, experience, host selection, parasitoids, *Cotesia marginiventris*, *Apanteles marginiventris*, *Spodoptera frugiperda*, fall armyworm, Hymenoptera, Braconidae, Lepidoptera, Noctuidae.

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## INTRODUCTION

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), is an important pest of annual row crops and pastures in the United States. Ashley (1979) lists 53 species of parasitoids that attack the FAW. *Cotesia* (=*Apanteles*) *marginiventris* (Cresson) is one of the parasitoids most frequently recovered from field-collected FAW larvae (Ashley, 1979). Also, this solitary larval parasitoid primarily attacks first and second instar larvae, killing the larvae when they reach the 4th instar (Ashley et al., 1982). For these reasons, this parasitoid appears to have considerable potential as a biological control agent. Effective biological control efforts against the FAW with this or other entomophages will require augmentative approaches such as environmental manipulation or periodic releases (Lewis and Nordlund, 1984).

Successful augmentative approaches in biological control must be based on an understanding of the host-selection behavior of the parasitoid involved, the various stimuli that may influence the behavior, and how the various stimuli function (Vinson, 1976). The host-selection behavior of parasitoids may be much more sophisticated than early studies indicate, in that learning, a well-documented phenomenon in insects (Arthur 1966, 1967, 1971; Gould and Gould, 1981; Taylor, 1974; van Lenteren, 1981; Vinson et al., 1977), appears to play a much greater role than earlier believed.

Loke et al. (1983) describes the host-finding behavior of *C. marginiventris* on corn plants and reports that parasitization was stimulated by FAW damage. Loke and Ashley (1984a) found that *C. marginiventris* initiates host-seeking behavior in response to both plant damage and host-insect related materials. They also demonstrated that this parasitoid responds to kairomones from the frass of FAW larvae (Loke and Ashley, 1984b).

This study was designed to determine the stimuli that influence the host-selection behavior of *C. marginiventris* and the role that previous experiences of the parasitoid may play in that behavior.

## METHODS AND MATERIALS

The studies were conducted using *S. frugiperda* (J.E. Smith) larvae, reared according to the procedure described by Perkins (1979), as hosts. *C. marginiventris* were reared according to the procedure described by Lewis and Burton (1970) for *Microplitis croceipes* Cresson.

The arena for observation of the parasitoid behavior (except for the antenna touch test) consisted of a frosted glass table, lighted from underneath with four 15-W cool-white fluorescent bulbs. The bioassay room was otherwise unlighted. Parasitoids and the materials to be examined were placed in the uncovered bot-

tom of a 10-cm-diam glass Petri dish located on the table and observed undisturbed.

Females of *C. marginiventris* were provided "experience" by placing five females of the age group to be tested in a 125 × 25-mm glass tube with 25 first and second instar host larvae feeding on pieces of corn leaves or other material, as indicated, for 3 hr immediately prior to the tests. An experience of 18 hr was provided in one antennal touch test (as described below). Inexperienced females of the same group and age, which had no previous contact with hosts, kairomones, or plant materials, were taken directly from holding cages.

## RESULTS

*Response to Chewed Corn Leaves.* The response of experienced and inexperienced females 1–2, 4–5, 6–7, and 8–9 days of age, to corn leaves chewed by fall armyworm larvae and unchewed control leaves was monitored. The chewed leaves were obtained by placing young leaves in a 125 × 25-mm glass ignition tube with first and second instar fall armyworm larvae for 2–3 hr. One each of a chewed and control leaf sample (2 cm in diameter) was placed in the observation arena with a female parasitoid. The parasitoid's behavior was observed for 15 min. The time spent resting, number of visits to each leaf sample, and duration of each visit were recorded. For each age group, the observations of the experienced and inexperienced individuals were alternated. This experiment was conducted with six experienced and six inexperienced females per age group.

The mean time spent at rest by the experienced and inexperienced parasitoids of various ages is shown in Figure 1. The experienced females clearly spent less time resting, although the difference was not significant for the 1- to 2-day-old females. The experienced female parasitoids preferred chewed leaves (Table 1). With the exception of the 1- to 2-day-old individuals, experienced parasitoids made significantly more visits to the chewed leaves and spent significantly more time examining chewed samples than unchewed samples. Inexperienced parasitoids exhibited no significant difference in their preference for chewed or unchewed leaves.

*Response to Larval Silk.* Pieces of filter paper 1 $cm^2$ were wrapped with 100 rotations of silk from first and second instar larvae by suspending a larva from the paper by its silk strands and rotating the paper as the larva spun. The larvae were never allowed to touch the paper directly. Clean 1-$cm^2$ pieces of filter paper were used as controls. Individual experienced and inexperienced parasitoids (1, 2, 3, 4, and 10 days of age) were exposed to both silk-wrapped and control papers in the observation arena and their behavior monitored. Six experienced and six inexperienced parasitoids were tested. In this test, visits by

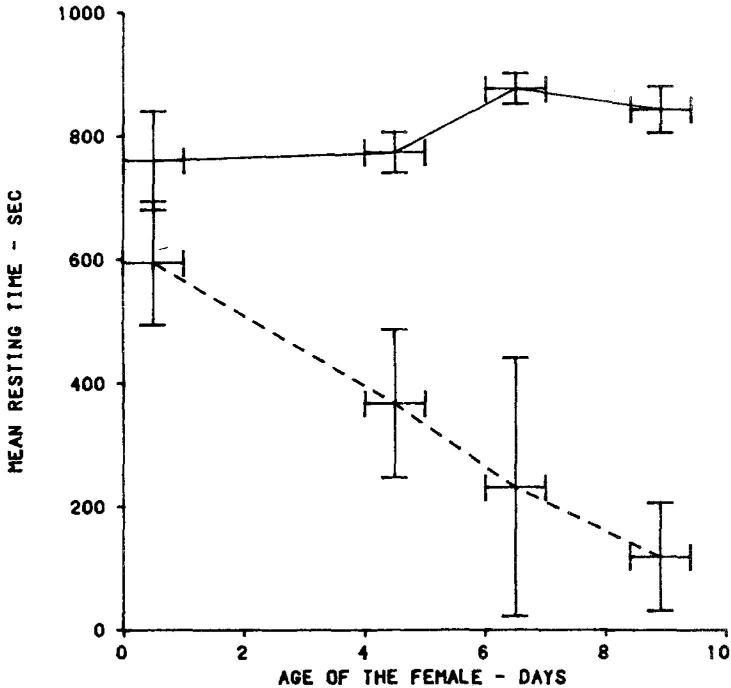


FIG. 1. Mean time spent in rest by experienced (----) and inexperienced (——) *C. marginiventris* females of various ages when exposed to a choice of unchewed leaves and leaves previously chewed by *S. frugiperda.*

the female to the filter papers with silk often resulted in a characteristic searching examination with their antennae. Therefore, data on the percent visits resulting in this examination were collected and considered a good measurement of the response to silk.

The data presented in Table 2 show that the percent of visits with antennal examination and mean time of the visits were not significantly different for inexperienced females. However, for the experienced females of all age groups, these responses to the silk-wrapped papers were significantly higher than to the control papers.

*Response to Larval Frass.* The response of female parasitoids to larval frass was tested in a manner similar to that used for larval silk. Fresh frass collected from fall armyworm larvae feeding on corn leaves was smeared on 1-$cm^2$ papers with a camel's hair brush. The parasitoids, 2–3 days old, were exposed individually in the observation arena to a pair of frass-contaminated and control papers for 10 min. The test was replicated with 10 experienced and 10 inexperienced parasitoids.

TABLE 1. COMPARATIVE RESPONSES OF EXPERIENCED AND INEXPERIENCED FEMALES OF *C. marginiventris* TO CHEWED AND UNCHEWED CORN LEAVES BY FALL ARMYWORM LARVAE[a]

| | Inexperienced | | Experienced | |
|---|---|---|---|---|
| Age of female (days) | Unchewed | Chewed | Unchewed | Chewed |
| Mean time of visit (sec). | | | | |
| 1-2 | 1.5a | 4.2a | 24.2b | 36.1b |
| 4-5 | 5.3a | 22.14a | 40.2c | 118.0b |
| 6-7 | 0.0a | 0.0a | 15.7c | 44.3b |
| 8-9 | 3.8a | 0.0a | 31.6c | 57.5b |
| Mean no. of visits | | | | |
| 1-2 | 0.2a | 0.4a | 2.1b | 2.8b |
| 4-5 | 0.3a | 1.2a | 2.3c | 8.0b |
| 6-7 | 0.0a | 0.0a | 5.2c | 10.3b |
| 8-9 | 0.2a | 0.0a | 3.7c | 8.3b |

[a]Means within the same experienced or inexperienced heading of the same horizontal rows followed by different letters are significantly different ($P = 0.05$) as determined by ANOVA. Means from six replications.

TABLE 2. COMPARATIVE RESPONSES OF EXPERIENCED AND INEXPERIENCED FEMALES OF *C. marginiventris* TO SILKS COLLECTED FROM 1ST AND 2ND INSTAR FALL ARMYWORM LARVAE[a, b]

| | Inexperienced | | Experienced | |
|---|---|---|---|---|
| Age of female (days) | Control | Silk | Control | Silk |
| Mean time of visit (sec.) | | | | |
| 3 | 1.5a | 15.0a | 5.0a | 52.5b |
| 4 | 1.9a | 10.5a | 4.2a | 52.5b |
| Percentage of visits with examination | | | | |
| 1 | 10.7a | 6.3a | 4.2a | 46.0b |
| 2 | 5.8a | 5.2a | 7.9a | 49.3b |
| 3 | 1.5a | 8.3a | 5.4a | 66.3b |
| 4 | 4.4a | 18.2a | 16.4a | 41.4b |
| 10 | 5.8a | 19.5a | 6.4a | 58.7b |

[a]Results of six replications for each age group.
[b]Means within the same experienced or inexperienced heading of the same horizontal rows followed by different letters are significantly different ($P = 0.05$) as determined by ANOVA.

With inexperienced females, percent visits with antennal examination (control 3.6, frass 5.3) or mean time of examination of the frass-contaminated and control papers (control 2.6, frass 8.2 sec) were not significantly different. In the case of experienced females, both the percent visits with examination (control 7.3, frass 43.2) and time of examination of frass-contaminated papers (control 5.6, frass 38.4 sec) were significantly higher ($P < 0.05$) than the control papers.

*Antenna Touch Test.* In this test, the response of individual female parasitoids, with various types of experience, was evaluated when their antennae were touched directly with host larvae, out of context with any other stimulus. The individual female was isolated in a 2-dr shell vial plugged with a piece of glass wool. Two fine wires were fashioned into a Y-shape at the end of a long, slim rod. By placing a first or second instar larva snugly in the Y-end of the rod so it was unable to escape, the observer was able to insert the larva through the glass wool plug and manipulate it so as to lightly touch the parasitoid's antennae, while avoiding other contact. Responses of the parasitoid during approach of the larva and after touch were carefully observed and classified into one of two categories: (1) excitement (faster walking, examination, irritation expressed by jumping) and (2) direct attack after the contact. Each experimental group consisted of six females. These females were first tested in the inexperienced state, and again after they were provided the experience of exposure, for 3 hr, to host-plant leaves, host larvae, hemolymph, or exuviae of host larvae. Each individual was touched 10 times, and the results were expressed as percent of times they exhibited each response.

Most of the inexperienced parasitoids exhibited excitement upon antennal contact with the larvae, but practically none attacked the host (Table 3). However, after being exposed to host larvae, frass, or exuviae, the percentage of females exhibiting the attack response increased significantly. Exposure to leaves of host plants with no larvae did not significantly increase the percentage of attacks. Some indications of change in behavior, although not statistically significant, were provided by the experience with *Amaranthus* leaves. This exposure to plant material may affect subsequent responses to plant material, but this was not tested in this study. The parasitoids experienced by contact with mature host larvae continued to demonstrate an increased level of attack after being isolated in empty vials for 24 hr (excitement 56.7, attack 46.7).

*Number of Contacts before Attack.* The purpose of this test was to determine how the experience of encounters and attacks of host larvae influenced subsequent attack behavior. A 6 × 2-cm piece of corn leaf with five first–second instar host larvae on it was placed in the bioassay arena, and one inexperienced female was introduced. The number of contacts with host larvae before each attack was monitored for 10 attacks. Each larva that was parasitized was immediately replaced by a new, unparasitized host. The test was replicated 10 times with 1- to 2-day-old and 4- to 5-day-old parasitoids.

TABLE 3. COMPARATIVE RESPONSES OF FEMALES OF *C. marginiventris* TO ANTENNAL TOUCH OF HOST LARVAE BEFORE AND AFTER INDICATED EXPERIENCES

| Age of female parasite (days) | Material provided for experience | Responses (%)[a,b] Inexperienced: Excitement only | Inexperienced: Attack | Experienced: Excitement only | Experienced: Attack |
|---|---|---|---|---|---|
| 1-2 | 1st & 2nd instar host larvae | 93.3a | 1.7c | 46.7b | 48.3b |
| 1-2 | Mature host larvae | 91.7a | 0.0c | 56.7b | 38.3b |
| 3-4 | 1st and 2nd instar host larvae | 90.0a | 0.0c | 53.3b | 41.7b |
| 3-4[c] | 1st and 2nd instar host larvae | 93.3a | 1.7d | 36.7b | 57.7c |
| 2-4 | Fresh host larvae frass (corn diet) | 50.0a | 5.0c | 73.3b | 15.0c |
| 2-4 | Mature host larvae hemolymph | 90.0a | 8.3c | 60.0b | 36.7b |
| 2-4 | Host larvae exuviae (varying instars) | 81.7a | 1.7d | 53.3b | 21.0c |
| 4-5 | Corn | 90.0a | 0.0b | 93.3a | 1.7b |
| 4-5 | *Amaranthus* spp. | 96.7a | 3.3b | 76.7a | 18.3b |
| 4-5 | Bermudagrass | 86.0a | 1.7b | 86.0a | 3.3b |

[a] Results of six replications.
[b] Means in the same horizontal rows followed by different letters are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test.
[c] Provided 18-hr contact rather than usual 3 hr.

The mean number of contacts prior to the first attack is approximately five for both age groups, then drops progressively for the next three attacks (Figure 2). These results demonstrate that the experience of encountering and attacking hosts increases the efficiency of subsequent attacks by the parasitoid. There is a slight rise in the number of contacts before each attack after the fifth attack, perhaps because of an epideictic pheromone on the leaf substrate or because of reduced oviposition pressure of the female.

*Discrimination of Parasitized Hosts.* In test A, the objective was to assess the influence of experience on discrimination between parasitized and unparasitized hosts. Five first-second instar host larvae were placed on a piece of corn leaf as in the previous experiment, two parasitoids were released in the arena, and the attacks by the parasitoids were observed. At the end of 10 min, the parasitoids were replaced with a new pair of females. The observations were repeated with five pairs of parasitoids, at 10-min intervals each, for experienced and inexperienced females. This test was replicated four times.

There was no significant difference in the number of attacks made by successive pairs of inexperienced females, but the number of attacks by experienced

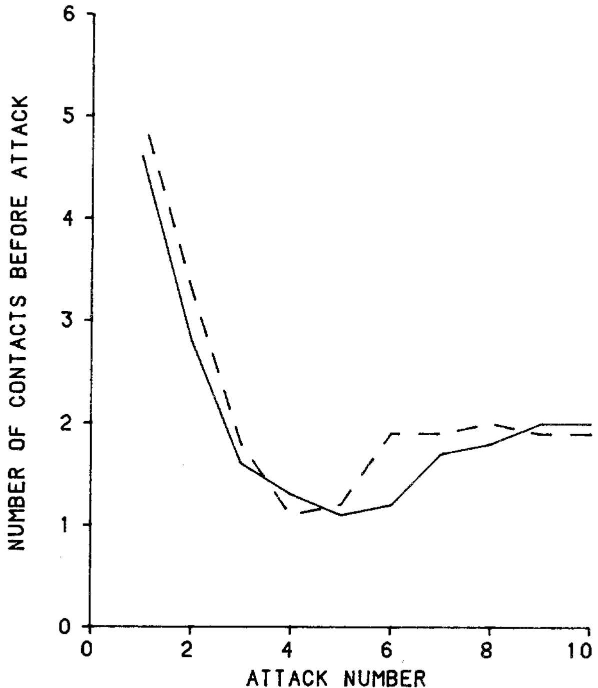


FIG. 2. Mean number of contacts with host larvae (*S. frugiperda*) before attack by *C. marginiventris* females of two ages (——, 1- to 2-day-old females; ----, 4- to 5-day-old females).

females decreased significantly, indicating their ability to detect the previous parasitization and to reject parasitized hosts (Table 4). The reason for the lower initial number of attacks by the experienced females is not known.

In test B, the objective was to determine the influence of previous parasitization on the number of contacts prior to parasitization and to examine the effect of experience on this behavior. Four groups of 10 females were prepared. Two groups were provided experience on first and second instar host larvae, and the other two groups were not experienced. Five first–second instar host larvae, all either unparasitized or previously parasitized, were placed on a 6-$cm^2$ piece of corn leaf and exposed to parasitoids in the bioassay arena. Each group of the experienced and inexperienced females was exposed to a set of either parasitized or unparasitized larvae in this manner and monitored for 10 min to determine the number of contacts with larvae that resulted in attack for each category. The attacked larvae were immediately replaced by unparasitized or parasitized larva, as appropriate. The entire test was conducted for both 1- to 2-day-old and 4- to 5-day-old parasitoids.

There was no significant difference in attacks of parasitized and unpara-

TABLE 4. COMPARATIVE OVIPOSITIONAL RESPONSES OF EXPERIENCED AND INEXPERIENCED FEMALES OF *C. marginiventris* WHEN EXPOSED IN SUCCESSIVE PAIRS TO A GROUP OF 5 HOST LARVAE[a]

| | Mean number of attacks | |
|---|---|---|
| | Inexperienced | Experienced |
| 1st pair | 7.1a | 4.9a |
| 2nd pair | 7.5a | 2.9b |
| 3rd pair | 8.9a | 1.2bc |
| 4th pair | 5.9a | 1.2bc |
| 5th pair | 5.3a | 0.4c |

[a] Means in the same vertical column followed by different letters are significantly different ($P = 0.05$) as determined by Duncan's multiple-range test.

sitized host larvae, measured either by percentage of contacts leading to attack or the actual mean number of attacks by inexperienced females of either age (Table 5). However, for the experienced parasitoids, there were significant differences in both percentage of contacts leading to attacks and mean number of attacks. These results further demonstrate the importance of learning in discrimination of parasitized host larvae and that discrimination occurs prior to attack.

TABLE 5. COMPARATIVE NUMBER OF CONTACTS OF HOST LARVAE LEADING TO ATTACK BY EXPERIENCED AND INEXPERIENCED FEMALES OF *C. marginiventris* FOR PARASITIZED AND UNPARASITIZED HOSTS[a]

| Age of female (days) | Inexperienced | | Experienced | |
|---|---|---|---|---|
| | Unparasitized larvae | Parasitized larvae | Unparasitized larvae | Parasitized larvae |
| Attacks after contact (%) | | | | |
| 1-2 | 83.3a | 82.1a | 80.7a | 56.9b |
| 4-5 | 61.8a | 73.0a | 71.6a | 32.8b |
| Mean number of attacks | | | | |
| 1-2 | 11.8a | 11.6a | 11.0a | 8.4b |
| 4-5 | 5.8a | 6.1a | 9.6a | 4.9b |

[a] Means within the experienced and inexperienced headings of the same horizontal rows followed by different letters are significantly different ($P = 0.05$) as determined by ANOVA. Means of 10 replications.

## DISCUSSION

Experienced *C. marginiventris* females of all ages exhibited strong and consistent responses to chewed leaves, silk, and frass of *S. frugiperda* larvae, as measured by the mean number of visits to the material and time of examination. Although the experiments described in this paper do not conclusively demonstrate the chemical nature of the stimuli involved, the work of Loke and Ashley (1984) and Weseloh (1976, 1977) would indicate that this is the case. Inexperienced females showed little or no response to these stimuli. Based on the antennal touch test, 3 hr of contact with host larvae of various ages, frass, exuviae, or hemolymph provided the necessary experience for the parasitoids to exhibit significantly increased attack responses when their antennae were touched with host larvae. Prior to the experience, these parasitoids exhibited excitement when touched by the larvae but did not respond with a properly coordinated attack. Prior experience of the parasitoid with only host-plant material did not provide for an increase in this attack behavior when the females were touched with the host larvae.

These results demonstrate that effective response of *C. marginiventris* females to the stimuli that mediate host selection involves learned behavior acquired from contact either with feeding host larvae or from contact with the silk, frass, exuviae, or other stimuli sources. In addition, the experienced females are more active and spend significantly less time at rest, particularly as they increase in age. The number of contacts with host larvae required prior to attack decreases with the number of attacks by the parasitoid.

One would expect the possibility of habituation where the parasitoids were exposed to silk, frass, and exuviae in the absence of host larvae for reinforcement, but no negative effects were detected under the experimental conditions used. The results, therefore, indicate the possibility that the change, as a result of these experiences, was more of a physiological nature at the sensory mechanism level versus an associative learning effect. Habituation and similar effects may come into play under field conditions where a fuller array of behavior interactions is permitted.

The results further show that discrimination between parasitized and unparasitized host larvae is a learned response similar to that demonstrated for other parasitoids (van Lenteren 1981). The discrimination was demonstrated to occur primarily at the time of initial contact of the host and prior to attack.

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# DEFENSIVE SECRETIONS OF NEW ZEALAND TENEBRIONIDS:
## V. Presence of Methyl Ketones in *Uloma tenebrionoides* (Coleoptera: Tenebrionidae)

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**Abstract**—The defensive secretion of *Uloma tenebrionoides* (White, 1846) contains benzoquinone, 2-methylbenzoquinone (toluquinone), and 2-ethylbenzoquinone as in other tenebrionids, together with 2-methoxyphenol, pentadecene, heptadecene, 2-pentanone, 2-pentadecanone, 2-heptadecanone, heptadec-10-en-2-one and (Z)-nonadec-10-en-2-one. The latter four methyl ketones have not previously been identified in tenebrionid beetles, and the unsaturated ketones are novel arthropod chemicals.

**Key Words**—Benzoquinones, defensive secretion, 2-heptadecanone, heptadec-10-en-2-one, heptadecene, 2-methoxyphenol, nonadec-10-en-2-one, 2-pentadecanone, pentadecene, 2-pentanone, *Uloma tenebrionoides*, Coleoptera, Tenebrionidae.

## INTRODUCTION

*Uloma tenebrionoides* of the tribe Ulomini is endemic to New Zealand where it is commonly found in rotting pine and other logs. Although the defensive secretions of over 150 species of tenebrionids have been analyzed, those of only three other genera previously assigned to the tribe Ulomini have been studied (Tschinkel, 1975a). These other genera (*Gnathocerus, Alphitobius,* and *Tribolium*) are not closely related to *Uloma* and should be removed from the tribe Ulomini (Watt, 1974). Tschinkel (1975a) studied two species of *Uloma* from North America (where *U. tenebrionoides* does not occur). In each of these he found toluquinone, ethylbenzoquinone, and pentadecene. In this paper we report



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the identification of additional compounds, including saturated and unsaturated methyl ketones, in *U. tenebrionoides*. Defensive glands of this species were described and illustrated by Kendall (1974).

## METHODS AND MATERIALS

Adult specimens of *U. tenebrionoides* were collected in the North Island of New Zealand at four different locations. (Voucher specimens of these beetles were deposited in the arthropod collection at Entomology Division, DSIR, Auckland, New Zealand.) The exudates of 20–25 specimens were used for each analysis. The method of the defensive secretions and the initial column chromatography on Florisil columns were as described by Gnanasunderam et al. (1981b).

*Gas Chromatography (GC), Gas Chromatography–Mass Spectrometry (GC-MS)*. GC was carried out on a Varian 2700 gas chromatograph fitted with a flame ionization detector using nitrogen as the carrier gas. GC-MS was carried out on a Varian 2700 gas chromatograph coupled via a membrane separator (at 18°C) to an AEI MS30 mass spectrometer operated at 20 eV for low resolution and 70 eV for high resolution (using PFK as an internal reference). Helium was used as the carrier gas. Separations were performed on one or other of the following: a 2-m × 2-mm (ID) stainless-steel column packed with 15% (w/w) OV-17 on Chromosorb W-AW-HMDS (100–120 mesh), a 50-m OV-101 glass SCOT column (SGE Australia), a 50-m Carbowax 20M glass SCOT column (SGE Australia), or a 30-m bonded phase DB 1 fused silica column (J. & W. Scientific Inc., USA). Analysis on the nonpolar columns was started at 60° and raised to 200° at 2 or 4°/min, while the Carbowax column was programed from 60 to 180°C at 4°/min.

*Semipreparative GC*. Individual peaks were collected in stainless-steel traps packed with Pyrex glass wool coated with OV-101, as described in Young (1981). For [$^1$H]NMR, the samples were collected in glass capillary tubes (100 1 Microcap, Drummond Scientific, USA) using the collection methods described by Young et al. (1983). Hydrogenation was carried out over Adams catalyst (Stanley and Kennet, 1973) in sealed capillary tubes.

*Nuclear Magnetic Resonance Spectra*. These were recorded on a 200-MHz Varian XL-200 spectrometer in deuterated chloroform. Chemical shifts are reported in ppm relative to tetramethylsilane as internal standards.

*Thin-Layer Chromatography (TLC)*. TLC was carried out on 5% silver nitrate–silica gel G (w/w) layer (200 × 100 × 0.2 mm). The solvent system used was 10% diethylether–pentane (v/v).

*Standard Compounds*. Standard samples of benzoquinone, toluquinone and 2-methoxyphenol were obtained from Merck chemicals, and hydrocarbons from Supelco Inc. 2-Ethylbenzoquinone, 2-pentadecanone and (*E*)- and (*Z*)-nonadec-

10-en-2-one were synthesized by Ms. Y.Y. Huang (University of Calgary, Alberta, Canada).

## RESULTS

The total secretion was chromatographed on a DB 1 bonded phase capillary column (Figure 1) and was further separated on a Florisil column into hydrocarbon (pentane), carbonyl (5% diethylether–pentane), alcohol (10% diethylether–pentane) and polar (20% diethylether–pentane) fractions.

Analysis of the hydrocarbon fraction by GC on both polar and nonpolar columns revealed the presence of a major (peak 8) and a minor peak (small contributor to peak 9). GC-MS of the two compounds represented by these peaks gave molecular ions at *m/z* 210 for the major component and *m/z* 238 for the minor component, together with fragmentation patterns typical of straight chain alkenes. The mass spectra and retention times were identical with those of authentic 1-pentadecene and 1-heptadecene.

To the human nose, the characteristic odor of *U. tenebrionoides* was as-

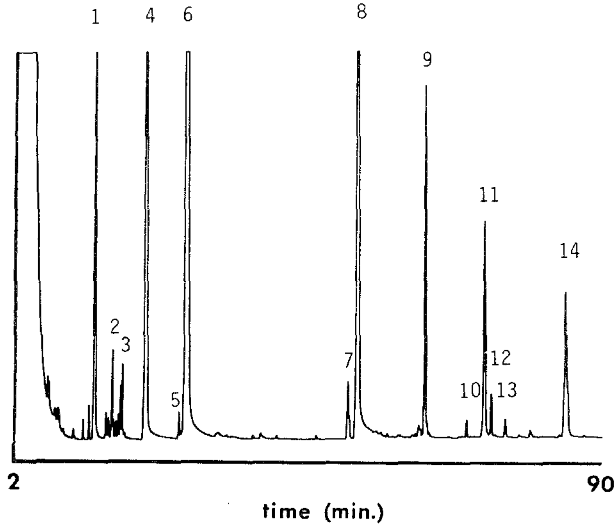


FIG. 1. Gas chromatography of the total secretion of *U. tenebrionoides* chromatographed on a DB 1 fused silica capillary column using He as carrier gas (25 cm/sec) and temperature programed from 60 to 200° at 4°/min. 1 = 2-pentanone, 2 = benzoquinone, 3 = 2-methoxyphenol, 4 = 2-methylbenzoquinone (toluquinone), 5 = unknown, 6 = ethylbenzoquinone, 7 = unknown, 8 = pentadecene, 9 = 2-pentadecanone + heptadecene, 10 = unknown, 11 = 2-heptadecanone, 12 = heptadec-10-en-2-one, 13 = unknown, 14 = (*Z*)-nonadec-10-en-2-one.

sociated with the carbonyl fraction. GC of this fraction showed several peaks. The mass spectrum of component A (peak 1) showed a molecular ion at *m/z* 86 and a base peak at *m/z* 43 with fragmentation ions at *m/z* 71 and 57, which was identical to the spectrum of authentic 2-pentanone. Its identity was confirmed by comparison of their retention times.

GC-MS of B (peak 9) gave a molecular ion at *m/z* 226 and intense ions at *m/z* 43 and 58, while [$^1$H]NMR spectrum contained an unsplit signal at $\delta$ 2.13, indicating that B was a methyl ketone. In the high field region of the [$^1$H]NMR spectrum there was a single triplet, also corresponding in area to a methyl group, representing the other chain end, i.e., B was an unbranched 2-alkanone. Accurate mass measurement data established B to have the composition $C_{15}H_{30}O$, i.e., to be 2-pentadecanone. This identification was confirmed by cochromatography of B and 2-pentadecanone on the Carbowax 20 M column, and the congruence of the mass spectra of B and the authentic 2-alkanone.

The mass spectrum of C (peak 11) also had intense ions at *m/z* 43, 58, and 71, again indicating a methyl ketone (Budzkiewicz et al., 1964) but with a molecular ion at *m/z* 254 and fragmentation ions at *m/z* 225, 196, 194, 168, 127, 113, and 85. This fragmentation pattern was similar to that of B but shifted 28 amu higher, suggesting C to be 2-heptadecanone. This identity was confirmed by comparison of its mass spectrum with that of a published spectrum of 2-heptadecanone (National Standards reference data base.)

The mass spectrum of D (peak 14), which also had intense peaks at *m/z* 43, 58, and 71, again suggested it to be a methyl ketone, but this time the base peak was at *m/z* 71. Mass measurement showed the molecular ion to have a formula $C_{19}H_{34}O$, which required D to be a monounsaturated or cyclic methyl ketone. [$^1$H]NMR analysis showed a sharp signal at $\delta$ 2.13 as required for a methyl ketone, but unfortunately the signal/noise ratio was too low to detect any multiplets in the vinylic region. A further sample of D was collected from the original carbonyl fraction on a Carbowax column and subjected to microhydrogenation. GC-MS analysis on the hydrogenated product had a molecular ion at *m/z* 282 and a fragmentation pattern which corresponded to the the published spectrum of 2-nonadecanone. This indicated that D was a nonadecen-2-one.

In order to establish the position of the double bond, D was again collected, ozononized, and the cleavage products analyzed by GC-MS. The single major volatile product of D gave a spectrum consistent with nonanal. This suggested that the double bond was at the C-10 position, and D was thus identified as nonadec-10-en-2-one. The mass spectrum of D was identical to the spectrum of authentic samples of (*E*)- and (*Z*)-nonadec-10-en-2-one. None of the GC columns used resolved the *E* and *Z* isomers, so TLC on $AgNO_3$–silica gel layer was used to distinguish between them. The $R_f$ value of D (0.47) matched that of the *Z* isomer. The $R_f$ value of the *E* isomer was 0.53, thus confirming D to be (*Z*)-nonadec-10-en-2-one.

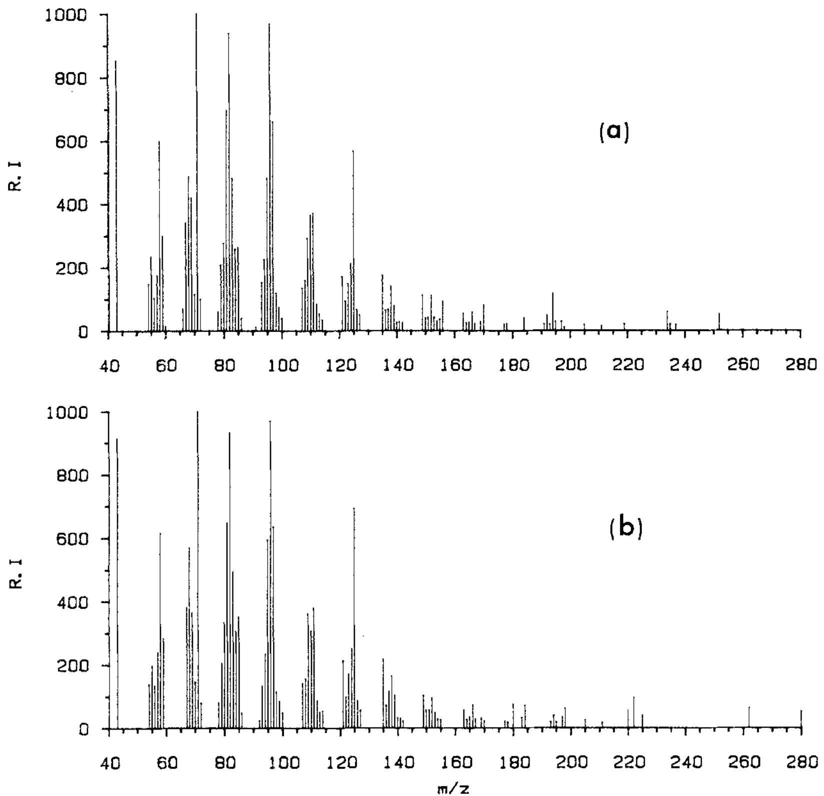


FIG. 2. Mass spectra of naturally occurring heptadec-10-en-2-one (a) and nonadec-10-en-2-one (b).

The mass spectrum of E (peak 12) was similar to that of D, but the molecular ion was at *m/z* 252 and the corresponding fragment ions were shifted to lower mass by 28 amu (Figure 2), indicating that E was heptadecenone. Analysis of the product of microhydrogenation of E by GC-MS revealed a single compound with a retention time corresponding to that of C and an identical mass spectrum. The main volatile ozonolysis product of E was identified by GC-MS as heptanel. Hence we again placed the double bond at the C-10 position and thus identified E as heptadec-10-en-2-one. Some other minor components in the carboryl fraction have not been identified.

GC analysis of the 10% ether–pentane fraction on polar and nonpolar columns did not show any detectable peaks, but analysis of the 20% ether–pentane fraction revealed the presence of six components. Compounds with retention times corresponding to peaks 2, 4, and 6 were identified as 1,4-benzoquinone, toluquinone, and ethyl-1,4-benzoquinone by comparing their mass spectra and

retention times with those of authentic materials. The mass spectrum of the compound represented by peak 3 gave a molecular ion at *m/z* 124 (100%) and fragmentation ions at *m/z* 109, 81, and 77. The mass spectrum and retention time of this compound were identical to those of an authentic sample of 2-methoxyphenol. The other minor compounds have not been identified.

## DISCUSSION

Methyl- and ethylquinones are well established components of the defensive secretions of a variety of arthropods (Blum, 1981, and references therein) and have been detected in all tenebrionids with defensive glands studied to date. The cooccurrence of 1-alkenes, such as pentadec-1-ene and heptadec-1-ene, in these secretions is also well precedented (Tschinkel, 1975a). Guaicol (2-methoxyphenol) has also been reported as a minor component in some polydesmoid millipedes (Blum, 1981, and references therein). More noteworthy is the presence of the ketones. None off these have been previously isolated from Coleoptera. Indeed, heptadec-10-en-2-one and nonadec-10-en-2-one are novel arthropod components, although 2-pentadecanone and 2-heptadecanone have been identified as alarm pheromones in the Dufour's gland secretions in the species of the stingless bee *Trigona* and in some species of the ant genus *Lasius* (Blum, 1981, and references therein) and also in the termite *Amitermes unidentatus* (Meinwald, 1978).

It is not readily apparent why some tenebrionid genera deviate from the usual quinone–alkene theme and produce additional chemicals. Some of these chemicals, such as 3-nonanone in *Eleodes beameri* (Tschinkel, 1975b), $\alpha$-pinene and limonene in *Artystona* (Gnanasunderam et al., 1981c), 4-methylhexan-3-one in *Amarygmus tristis* (Gnanasunderam et al., 1982), methyl-6-methyl salicylate in *Chrysopeplus expolitus* (Gnanasunderam et al. 1984), and now 2-pentadecanone and 2-heptadecanone in *U. tenebrionoides*, have been identified as alarm pheromones in social insects. It is possible that these chemicals function as cryptic alarm pheromones as suggested by Blum (1981).

Certainly the methyl ketones in *U. tenebrionoides* contribute to the characteristic odor of the insect when disturbed, which is a useful character for identifying this species (J.C. Watt, personal communication). We wish to draw attention to some circumstantial evidence which suggests that the odiferous defensive secretions of arthropods may be used by predators to locate them. In a recent study of the ecology of *Apteryx austalis* (Kiwi) in pine forests it was noted (R. Kleinpaste, personal communication) that gizzards and droppings of these birds commonly contained the remains of *U. tenebrionoides* and other insects known to possess odiferous defensive secretions such as the staphylinid of the genus *Thyreocephalus* (Gnanasunderam et al., 1981a) and the blattid *Platyzosteria novaeseelandia* (Benn et al., 1977). Nocturnally active, the kiwi has

poor eyesight but a well-developed sense of smell; it is the only bird known to possess external nares at the tip of its bill (Reid and Williams, 1975). It is known that the kiwi searches for its food using its nostrils (Reid and Williams, 1975), and Wenzell (1968) has demonstrated that it can locate its food by olfaction alone. Unfortunately the lack of behavioral observations at present precludes the understanding of the roles of these additional chemicals that supplement the quinonic secretions of these tenebrionid beetles.

In terms of their biosynthesis it appears most likely that the ketones of *U. tenebrionoides* are produced by the beetle (and not sequestered from some food stuff) by chain elongation of a fatty acid to yield a $\beta$-keto acid which then suffers decarboxylation. Oleic acid is thus visualized as the precursor of (Z)-10-nonadecen-2-one. Although this process has not been demonstrated in *U. tenebrionoides* it is known that arthropods are capable of building up long-chain hydrocarbons and ketones from small molcules (Duffey, 1976), and fatty acid chain elongation and decarboxylation have been demonstrated in the blattid *Periplaneta americana* (Condrad and Jackson, 1971) and the millipede *Graphidosteptus tumilporus* (Oudejans and Zandee, 1973).

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# EVALUATION OF PLANT CONSTITUENTS ASSOCIATED WITH PECAN PHYLLOXERA GALL FORMATION[1-4]

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**Abstract**—The weights of pecan *Carya illinoensis* Koch galls caused by several species of *Phylloxera* (Homoptera: Phylloxeridae) were negatively correlated with leaf and nut weights and nut production. Several allelochemicals (isoquercitrin, juglone, and 2 proanthocyanidins) were isolated from galls, and their antibiotic potentials were estimated, based on their toxicity to the bacteria *Pseudomones maltophilia* (Hugh et Ryschenkow). Pecan proanthocyanidins (condensed tannins) were characterized for the first time, and their stereochemistry was elucidated. The protein and total sugar contents of total leaves and leaf surface washings were determined. The leaf surface sugar content was very low, suggesting that the puncturing strategy of this insect may be for the purpose of finding sugars. The plant growth hormones gibberellic acid, zeatin, zeatin riboside, kinetin, indole acetic acid, and abscisic acid were found in pecan leaves, stems, and their galls. Gibberellic and abscisic acids were present in highest concentrations in all tissues, but lower in galled tissues, suggesting that increased biosynthesis by pecan plant growth regulators did not occur in response to insect attack.

**Key Words**—*Phylloxera* spp., Homoptera, Phylloxeridae, pecan, galls, allelochemicals, isoquercitrin, juglone, proanthocyanidins, plant hormones, gibberellic acid, zeatin, zeatin riboside, kinetin, indole acetic acid, abscisic acid, antibiotics.

[1] Juglandales: Juglandaceae.
[2] Homoptera (Heteroptera): Phylloxeridae.
[3] Mention of a commercial or proprietary product in this paper does not constitute endorsement of this product by USDA or Mississippi State University.
[4] Mississippi Agricultural and Forestry Experiment Station Journal Article 5830.

## INTRODUCTION

Five species of *Phylloxera* (Homoptera: Phylloxeridae) have been reported on pecan: pecan phylloxera *Phylloxera devastatrix* Pergande, pecan leaf phylloxera *P. notabilis* Pergande, *P. perniciosa* Pergande, southern pecan leaf phylloxera *P. russellae* Stoetzel, and lesser pecan leaf phylloxera, *P. texana* Stoetzel (Stoetzel, 1981). *P. devastatrix* attacks the stem, twigs, leaf petioles and mid-ribs, catkins, and fruits (Carpenter et al., 1979); *P. notabilis, P. russellae,* and *P. texana* attack the leaflets (Boethel et al., 1976; Stoetzel and Tedders, 1981; Stoetzel, 1981).

Probably the most damaging phylloxera, *P. devastatrix,* whose life cycle was described by Boethel and Ezell (1977), has the potential to devitalize susceptible trees and to destroy an entire pecan crop. This species is common in the delta and other sections of Mississippi and is the subject of the study reported herein.

The overwintering stage of the pecan phylloxera is an egg enclosed within the body of a dead female from the previous season. It remains sheltered in bark crevices or under lichens during the winter months. The egg remains dormant from early summer until the following spring (or about 10 months). Just prior to bud break and for several weeks thereafter, the eggs hatch into nymphs which crawl onto the developing terminals and soon thereafter settle down to feed, thereby eliciting gall formation by the tree.

The effects of gall-forming insects on plants and trees are generally important because the galls can reach weights in excess of 300% of the infested leaf (Skuhravey et al., 1980), thus diverting energy normally used for other plant activities such as fruit production.

In this study, several subjects were addressed. The weights of galls were recorded and related to leaf and nut weights and to nut production. Several so-called secondary plant constituents (allelochemicals) were found in the galls, as well as in the leaves, and their antibiotic potencies against a bacteria were evaluated. Bacteria were utilized because no insect bioassay could be performed. However, it has been established that compounds toxic to bacteria are often toxic to insects and thus a predictor of plant resistance to insects (Elliger et al., 1980). Pecan gall proanthocyanidins (condensed tannins) were characterized for the first time. Because pecan phylloxera are known to feed beneath the leaf and stem surfaces, the surface and total leaf protein and sugar contents were determined to reconcile, if possible, the established feeding patterns. Also pecan leaves, stems, and their galls were analyzed for their plant hormones contents. Byers et al. (1976) reported that larvae of the midge *Janetiella* sp. cause galls at the base of young needles of pinyon *Pinus edulis* (Engelm). Bioassays of extracts from these galls contained as much as 17 times more auxin activity and as much as 21 times more gibberellin-like activity per needle than extracts from normal needles of the same age.

Although elucidation of gall initiation, growth, and resistance factors is the ultimate goal, only descriptive work was possible in this present study because procedures for rearing insects and initiating gall development in the laboratory have not been developed.

## METHODS AND MATERIALS

*Effects of Gall Formation on Leaf and Nut Weights.* From late June through mid-October of 1983 pecan shoots of var. Success and Stuart trees located near Tchula and Greenwood, Mississippi, that possessed intermediate and heavy (approximately two and six galls per shoot) gall formations were harvested. Leaves, nuts, and galls were counted, and their weights were recorded; up to 109 shoots were employed in the several tests, and shoots of ungalled trees were also harvested for comparison. Standard statistical analyses (Duncan's multiple-range test) were performed.

*Isolation of Isoquercitrin from Early Summer Pecan Leaf Galls.* Green, chlorophyll-containing galls of var. Stuart (30.7 g, fresh weight) were extracted by grinding in a blender with chloroform–methanol, 2:1. The gall residue was then similarly extracted with 70% aqueous methanol to give 5.1 g of total solids. The solids were dissolved in 70% aqueous methanol and chromatographed on a Sephadex LH-20 column (75 cm long; 5 cm ID; solvent, 70% aq methanol). Two-dimensional TLC (solvent 1, *t*-BuOH–HOAC–$H_2O$: 3:1:1; solvent 2, 15% aq HOAC) of the total 70% aq methanol column eluate followed by visualization of the chromatogram with diphenyl boric acid–ethanol amine complex revealed one major and two minor flavonoids plus condensed tannin. Rechromatography of the flavonoid fraction with the same solvent yielded a yellow orange band (yield: 0.4 g, 1.3%) of which a portion was hydrolyzed with boiling 1 N HCl. The hydrolysate was extracted with ethyl acetate, and the remaining aqueous phase was evaporated to dryness and extracted with pyridine for investigation of the sugar. CI-MS (solid probe, $CH_4$) of the ethyl acetate fraction gave a M+1 ion: 303, quercetin ($C_{15}H_{10}O_7$) = 302. CI-MS of the sugar residue gave *m/e* 163, 145, 127, 108; compared to glucose (163, 145, 127, 108). TLC (solvent 1) of the unhydrolyzed band gave a spot coincident with isoquercitrin. UV–visible spectra in methanol and to which the spectral shift reagents (NaOMe, $AlCl_3$, $AlCl_3$ + HCl, NaOAC, and NaOAC + $H_3BO_3$) were added were identical with isoquercitrin.

*Structure of Pecan Leaf Condensed Tannin (Proanthocyanidin).* Leaf tissue was collected in July from eight var. Stuart trees, freeze dehydrated, and ground to a powder. The yield from 2 kg of fresh leaves was 994 g (49.7%). Chlorophylls and nonpolar components were removed by extraction 3X with cyclohexane–ethyl acetate–HOAC (500:500:1). The residue was extracted with 70%

aqueous acetone. Evaporation of the extract yielded 15.2 g of a crude tannin (1.6%). A portion of the solids (5 g) was chromatographed on a Sephadex LH-20 column (75cm long; 5 cm ID; solvent, 70% aqueous acetone). Subfractions which were similar when evaluated by TLC (polyamide 75% aq methanol) were combined and gave 10 fractions. By heating an aliquot of each fraction with *n*-butanol–HCl (95:5) to develop anthocyanidin pigments, it was determined that most of the tannin (and some coincidental flavonoids) were eluted with 70% aqueous acetone.

Fractions 3, 4, and 5, which were found to be high in condensed tannins, were combined because of their similar behavior on diagnostic TLC. They were rechromatographed under the previously described conditions (LH-20, 70% aq acetone) to yield a compact eluting band, which upon freeze-dehydration yielded 0.223 g of a red–brown powder. Acid hydrolysis of a small portion yielded a mixture that was chromatographed on polyamide TLC sheets with methanol and gave red spots coincident with standard cyanidin and delphinidin, presumptive for a condensed tannin. Fraction 10, also present at a relatively high concentration, was rechromatographed to yield a compact eluting band, and yielded 0.468 g of a red–brown powder upon freeze-dehydration.

$[^{13}C]$NMR analysis was performed with a Varian CFT-20 spectrometer at 20 MHz and 37°C using an 8K data table. The spectrum was obtained from a near-saturated solution (> 100 mg in 1.5 ml) of the proanthocyanidins in $D_2O$–$d_6$-acetone (1:1 v/v) using 45° pulses at 0.2-sec intervals during continuous proton decoupling. Spectrometer conditions and the assumption of the same $T_1$ and $\eta$ for similar aliphatic and aromatic carbons for comparing peak areas has been shown to be valid for proanthocyanidin polymers (Czochanska et al., 1979, 1980).

*Analysis of Condensed Tannins.* Condensed tannin analyses were performed on 70% aqueous methanol extracts of tissue. The chromophore was developed by boiling an aliquot 1 hr with *n*-butanol–HCl (95:5). The percent content was estimated by comparison with the color obtained at 550 nm from a purified cotton condensed tannin sample, the structure of which has recently been elucidated by Hedin et al. (1983).

*Analyses of Juglone.* Juglone analyses were accomplished using a technique developed by Hedin et al. (1979). Extractions were made from 1–10 g of fresh tissue by grinding in chloroform–methanol (2:1). The filtrate from three successive extractions of tissue was combined and concentrated to 25 ml by evaporation under vacuum at 50°C. A 1-ml aliquot was banded on a silica gel-G TLC plate and chromatographed with methylene chloride–pentane (1:3). Juglone was observed as a yellow–orange band at $R_f$ 0.40, scraped into a test tube, eluted from the silica gel with methylene chloride, filtered, and diluted to 10 ml for spectrometric analysis at 420 nm. For comparison, a standard curve was prepared with dilutions of 0.05–0.50 mg/10 ml of an authentic sample of juglone

(Aldrich Chemical Co., Milwaukee, Wisconsin). MS analysis showed that the fragmentation patterns of the commercial sample and that from pecan leaves were identical (Hedin et al. 1979, 1980).

*Antibacterial Bioassay.* Several compounds that had been isolated from pecan were screened for antibacterial activity against the pathogen *Pseudomonas maltophilia* (Hugh et Ryschenkow) with bactosensitivity disks (BBL). Each fraction to be tested was dissolved in an appropriate solvent (1 mg/20 $\mu$l and dilutions). A 20-$\mu$l aliquot of the solution was applied to a blank sensidisk, and the solvent was allowed to evaporate overnight. Three replications were employed for each sample. A suspension of each bacterium in a 0.85% saline solution was used to streak Petri plates (15 mm × 100 mm diameter) containing solidified tryptic–soy agar. The sensidisks were placed on the plates, no closer than 10–15 mm from each other (no more than seven disks per plate), and the plates were incubated overnight at 37°C. Degree of inhibition was determined by measuring the diameter of the clear zone (if present) around the disk.

*Analysis of Pecan Leaf Protein and Sugars.* For determination of leaf surface compounds, fresh Stuart var. leaves (5 kg) were harvested on July 7, washed 4 × with 10-liter portions of distilled water, the washes were combined, and allowed to evaporate to 50 ml at room temperature in a hood. An aliquot was used to determine percent total solids. Protein analysis was determined by the colorimetric procedure of Bradford (1976) and sugars were determined by the colorimetric anthrone procedure (test solution added to 10 ml of 0.05% anthrone and 1.0% thiourea in 72% aq $H_2SO_4$, boil 15 min, read absorptivity at 620 nm, compare with standard glucose). To determine total protein content, Stuart leaves harvested on June 26 were freeze-dehydrated, ground, and analyzed for Kjeldahl protein by standard AOAC procedures (14.115), and for sugars by the anthrone procedure.

*Analysis of Plant Hormones.* Extractions and analyses were performed by adaptations of the methods of Hardin and Stutte (1981). Replicates of 1 g of fresh leaves, stems, leaf galls, and stem galls from var. Desirable were gathered May 19 and ground with 10 ml of acetone; 5 ml of water was added, the extract filtered, and the acetone evaporated. The sample was filtered through celite, the pH of the solution was adjusted to 2.5, and then the hormones were extracted from the aqueous phase with diethyl ether. The ether was evaporated with a $N_2$ stream, and the hormones were resuspended in 80% aqueous methanol. The hormones (approximately 1% of the extract) were then chromatographed via HPLC on a $\mu$Bondapak $C_{18}$ cartridge column (Waters Associates) employing 0.5% acetic acid ($6 \times 10^{-3}$ M ammonium acetate)–acetonitrile (80:20), and, when required, methanol–acetonitrile (58:42). Detection was at 254 nm. Concentrations were determined with reference to known quantities of known compounds as furthur described.

*Verification of HPLC Maxima.* The compounds gibberellic acid, zeatin,

zeatin riboside, kinetin, indole acetic acid, abscisic acid, and indole butyric acid (Sigma Chemical Co., St Louis) were dissolved in 80% aqueous methanol, and adjusted to pH 2.2 to effect solution where required. An aliquot of each (5 $\mu$g) was chromatographed under identical conditions as were the unknowns.

Eluants from the maxima obtained during HPLC analyses of the plant extracts were collected, concentrated, methylated with diazomethane, and subsequently silylated at 60°C with a mixture of hexamethyl disilazane and trimethyl chlorosilane in pyridine. An aliquot was then analyzed via solid probe by EI-MS. Subsequently the reference compounds were methylated, silylated and analyzed by EI-MS in similar fashion. Mass spectral analysis of methylated, trimethylsilylated gibberellins and other compounds has been reported by Binks et al. (1969) and others.

## RESULTS AND DISCUSSION

*Effect of Gall Formation on Leaf and Nut Weights.* Because, as previously discussed, plant galls of many species can divert energy normally used for productive activities, recordings were made on several occasions during the summer and fall of 1983 of pecan leaf, nut, and gall counts and their respective weights to determine whether negative correlations between galls and fruit yield could be established.

On June 29, 30 terminals each of shoots with light, medium, and heavy gall formations of var. Success were harvested. The results, summarized in Table 1, show that at this relatively early stage, there were only minor differences in the number of nuts per shoot, even though the heavily galled group contained 11.6 times more galls (6.57 galls per shoot) than the light group.

On July 7, weights of leaves, nuts, and galls were recorded for each shoot of galled and nongalled trees, 13 shoots at Tchula and 16 at Greenwood. When the ratios of nut weight to leaf weight versus gall weight to leaf weight were plotted for the trees at the Tchula orchard, the output was a distribution of points with the general shape of a negative logarithmic curve. The $r$ values were sig-

TABLE 1. EFFECT OF GALL FORMATION ON EARLY SEASON NUTLET QUANTITY PER SHOOT; VAR. SUCCESS

| Gall incidence | Nuts[a] | Avg. nuts/shoot[b] | Galls[a] | Avg. galls/shoot |
|---|---|---|---|---|
| Light | 52 | 1.73b | 17 | 0.57a |
| Medium | 75 | 2.50a | 74 | 2.47b |
| Heavy | 61 | 2.03b | 197 | 6.57c |

[a] Per 30 shoots.
[b] Means followed by common letter did not differ significantly at the 0.05 level.

nificant at the 0.05 level for raw data (−0.698*), log ($X$ + 1) transform (−0.701*), log ($Y$ + 1) transform (−0.743*), and log ($X$ + 1) and log ($Y$ + 1) transform (−0.694*). The correlations at the Greenwood orchard appeared to be promising, but failed significance at the 0.05 level. At this stage, the data showed that intermediate nut production can be achieved with a gall weight to leaf ratio of 5–7%; however, in no recorded instance was the nut weight to leaf weight ratio above 10% with similar (10%) gall to leaf ratios.

On July 20, this test was repeated on a larger scale in which 109 shoots were harvested from Success trees with differing amounts of gall formation. The ratios of nut weight to leaf weight versus gall weight to leaf weight were plotted and also produced a distribution of points with the general shape of a negative logarithmic curve, but the $r$ value failed significance at the 0.05 level. However, some trends were apparent and were in general harmony with the previous study. They showed that a nut–leaf weight ratio of above 12.5% was not achieved at gall–leaf weight ratios above 5%. In this test, gall–leaf weight ratios of 0.54–1.20 were recorded in 8% of the shoots. Because fresh weights only were recorded and the galls contained a lower moisture content, these ratios, when corrected for moisture, approach the three fold ratios reported by Skuhravey et al. (1980) for gall midges.

On July 20, shoots from galled and ungalled Success and Stuart trees (total of 70) were harvested, and the weight of leaves, galls, and nuts were again recorded and statistically evaluated by the Duncan multiple-range test. Table 2, which summarizes the data, shows that the Stuart trees were evidently more resistant to attack by *Phylloxera* spp. because no galls were formed, and leaf and nut weights were higher. With the heavily and intermediate infested shoots from Success, leaf weights were decreased, and nut weight was zero when the gall/leaf weight ratio was 32%. Although a comparison of the effects of gall formation on leaf and nut weight might be confounded by somewhat different horticultural properties of two varieties, in this case the effects of galls are sufficiently great to overwhelm varietal differences.

TABLE 2. EFFECT OF GALL FORMATION ON MID-SEASON LEAF AND NUT WEIGHTS (AVERAGE WEIGHT/SHOOT)

| Incidence | Shoots | Gall weight[c] | Leaf weight | Avg. nut wt. per shoot |
|---|---|---|---|---|
| Heavy[a] | 30 | 2.85a | 8.90a | 0.00a |
| Intermediate[a] | 10 | 2.44a | 10.80a | 1.81a |
| Ungalled[b] | 30 | 0.00b | 17.47b | 2.06b |

[a] Var. Success.
[b] Var. Stuart.
[c] Means followed by a common letter did not differ significantly at the 0.05 level.

TABLE 3. EFFECT OF GALL FORMATION ON LATE SEASON NUT PRODUCTION AND WEIGHT (GREENWOOD)

| Incidence | Shoots | Avg. galls/shoot[a] | Avg. nuts/shoot | Avg. wt. of viable nuts per shoot (g) |
|---|---|---|---|---|
| Light or none | 30 | 0a | 2.80a | 68.09a |
| Intermediate | 30 | 1.97b | 170b | 58.55a |
| Heavy | 30 | 5.87c | 1.33b | 30.60b |

[a]Means followed by a common letter did not differ significantly at the 0.05 level.

In a final test in which shoots were gathered from the Greenwood orchard on October 11 and from the Tchula orchard on October 13, the number and weight of nuts and the number of galls per shoot were recorded and statistically evaluated by Duncan's multiple-range test. The data which are summarized in Table 3 indicate that in the Greenwood orchard, some trees remained free of galls while others had intermediate numbers (1.97 galls/shoot) and still others had heavy (5.87 galls/shoot) numbers. With the heavy incidence, nut population, nut weight, and total yield per shoot (30.60 g) were decreased. The average nut weight per shoot at the intermediate level (58.55 g) was slightly less than that of the control (68.09 g). In the Tchula orchard, all the trees showed galling, and the number of nuts per terminal and nut weights were much lower (most of the nuts had abscised in mid-season for unknown reasons). The total yield per shoot was higher in the intermediate (14.83 g) than in the heavier (7.83 g) galled trees.

*Isolation of Isoquercitrin from Pecan Leaf Galls.* Green, chlorophyll-containing galls of var. Stuart contained 1.3% isoquercitrin and two other flavonoids present in trace quantities. When hydrolyzed in 1 N HCl, the two flavonoids did not yield quercetin or any other recognizable flavonoid aglycone and were not furthur investigated. The tannin identification and biological evaluation work is discussed elsewhere in the text.

When isoquercitrin was tested for antibacterial activity to *P. maltophilia* (see Methods and Materials), growth was depressed, but not as strongly as with juglone and condensed tannin. Isoquercitrin has also been reported to inhibit tobacco budworm [*Heliothis virescens* (Fab)] larval growth; the $ED_{50}$ as percent of diet was 0.06% (Hedin et al., 1983). Thus, isoquercitrin is present in galls (and in leaves) in antibiotic concentrations, even though gall information is not prevented.

*Structural Features of Condensed Tannins.* Of the four criteria required for the gross structural elucidation of proanthocyanidin polymers, three were obtained using previously established [$^{13}$C]NMR spectroscopic techniques: (1) the

ratio of procyanidin [PC(1a)] to prodelphinidin [PD(1b)] units; (2) the stereochemistry of the heterocyclic ring of the monomer units; and (3) the number average molecular weight (mol wt). The fourth criterion, the structures(s) of chain-terminating flavan-3-ol unit, may be determined from GLC analysis of the products of degradation experiments but was not done in this study.

The [$^{13}$C]NMR spectra of fractions PT 3, 4, 5, and of PT 10 had a somewhat low signal-to-noise ratio due to limited amount of material and possibly because of some contamination from a carbohydrate impurity. Nevertheless, the general spectral patterns and chemical shifts were consistent with spectra obtained on previous 4–8 linked polyflavan-3-ol polymers (Czochanska et al., 1979, 1980). The apparent number average mol wt for both fractions was estimated as 1200 $\pm$ 200. The PD–PC ratio for both fractions was approximately 2:1, indicative that both fractions were of the same general structure. The ratio of the C-3 monomer unit signal to the terminal C-3 signal indicated an internal to terminal monomer unit composition of 4:1 consistent with the molecular weight (Table 4). The two fractions did not appear to possess the same stereochemistry because of several differences in the spectra (Foo and Porter, 1983). However, these could not be evaluated rigorously due to the high spectral noise and probable presence of impurities in the limited amount of material upon which the spectral measurements were conducted.

*Analysis of Juglone and Condensed Tannins.* Pecan leaves, stems, and their galls were analyzed for juglone and condensed tannins (Table 5). The content of these compounds was highest in leaves and the content in leaf galls was reduced, but tannins were increased in galled stems. The juglone content in the tissues was similar to that reported for early summer pecan leaves by Hedin et al. (1979), but much lower than that in cotton leaves (5–15%) (Hedin et al. 1983), for example. Juglone is present in the free form and has been shown to be a factor of disease resistance in pecan (Hedin et al., 1979, 1980). However, its content is not increased at the infection site (Hedin et al., 1980).

Leaves were also collected from trees at Greenwood, Mississippi, in early July 1983. Leaves from branches of Success pecan trees that were heavily galled contained 0.035 $\pm$ 0.006 mg/g juglone, while leaves from resistant trees (var.

TABLE 4. ESTIMATES OF STEREOCHEMICAL COMPOSITION AND MOLECULAR WEIGHT OF PROANTHOCYANIDIN POLYMERS

| Polymer | Mol wt[a] | C-3:C-3t | X *cis* | PD:PC |
|---|---|---|---|---|
| PT 3, 4, 5 (223 mg) | 1200 ± 200 | 3.5–4.8:1 | 0.93 | 63:37 |
| PT 10 (468 mg) | 1200 ± 200 | ~4:1 | 0.88 | 67:33 |

[a] Based on PD–PC percentages with average molecular weight per unit of 298.

TABLE 5. ANALYSES OF JUGLONE AND CONDENSED TANNINS IN PECAN LEAVES, STEMS, AND THEIR GALLS[a]

| Tissue | Amount (mg/g fresh wt) | |
|---|---|---|
| | Juglone | Tannins |
| Leaves | 0.065 ± 0.012 | 7.2 ± 1.6 |
| Stems | 0.017 ± 0.003 | 2.0 ± 0.4 |
| Leaf galls | 0.011 ± 0.003 | 3.2 ± 0.6 |
| Stem galls | 0.007 ± 0.002 | 4.0 ± 0.6 |

[a] Var. Desirable gathered May 19, 1983.

unidentified) free of galls contained 0.070 ± 0.006 mg/g juglone. Thus, some correlational evidence for juglone as a resistance factor was obtained.

*Antibiotic Compounds.* Juglone, condensed tannin, and isoquercitrin were bioassayed at 100 μg/disk using the bacteria *Pseudomonas maltophilia.* Their activities, expressed as zone width in millimeters, were: juglone, 14.0; condensed tannin, 13.0; and isoquercitrin, 7.0. Flavonoids and other so-called secondary plant constituents have been implicated as factors of resistance to insects (Feeny, 1968; Hedin et al., 1983). Elliger et al. (1980) examined 40 flavonoids for antigrowth activity toward the corn earworm, *Heliothis zea* Boddie. Waage and Hedin (unpublished data) tested 63 flavonoids for antibacterial activity and found that there were many instances in which compounds that were antibiotic to bacteria were also antibiotic (toxic) to insects. Therefore, compounds present in pecan tissue (and galls) could be antibiotic to *Phylloxera.* Although the compounds are much less active than insecticides, they exist in the plant in concentrations at least as high as that required for activity when constituted in laboratory diets and fed to larvae such as the tobacco budworm (Hedin et al., 1983).

*Analysis of Pecan Leaf Protein and Sugars.* The total content, as determined by analysis of the freeze-dehydrated leaf powder, for var. Stuart was: protein, 12.5 ± 0.8%; total sugars, 4.4 ± 0.4%. By washing 5 kg fresh leaves with water, 1.025 g of solids were obtained (0.021%). Of the total solids, 22.5% was protein and 0.06% was total sugars. Microscopic examinations have shown that *Phylloxera* nymphs feed in the phloem cells by puncturing between cuticular cells walls. Considering the low surface sugar content, the puncturing strategy may be for the purpose of finding sugars.

*Analysis of Plant Growth Hormones.* Chromatography by HPLC (see Methods and Materials) of pecan leaves, stems, and their galls each gave 12 maxima; six of the retention times were coincident in increasing order with gibberellic acid, zeatin riboside, zeatin, kinetin, indole acetic acid, and abscisic acid.

The identities of five of the maxima were confirmed by performing EI-MS

TABLE 6. PLANT GROWTH HORMONE CONTENTS OF PECAN LEAVES, STEMS, AND THEIR GALLS[a]

| Compound | No. | Maxima rt (min) | Content (μg/g) | | | |
|---|---|---|---|---|---|---|
| | | | Leaves | Stems | Leaf galls | Stem galls |
| Gibberellic acid | 1 | 1.7 | 9060 ± 65.0 | 698 ± 45.0 | 400 ± 35.0 | 345 ± 31.0 |
| Zeatin riboside | 2 | 2.2 | 8.5 ± 2.1 | 4.6 ± 1.0 | 6.4 ± 1.2 | 4.2 ± 0.6 |
| Zeatin | 3 | 2.4 | 3.3 ± 0.9 | 2.4 ± 0.6 | 1.7 ± 0.4 | 1.3 ± 0.3 |
| Kinetin | 5 | 4.0 | 11.5 ± 3.4 | 4.8 ± 0.8 | 3.9 ± 0.7 | 2.7 ± 0.3 |
| Indole acetic acid | 7 | 8.7 | 60.3 ± 7.8 | 41.4 ± 5.3 | 28.1 ± 4.5 | 13.3 ± 2.4 |
| Abscisic acid | 10 | 12.5 | 114.0 ± 10.2 | 82.1 ± 9.1 | 36.5 ± 6.5 | 16.7 ± 3.5 |

[a] Var. Desirable gathered May 19, 1983.

via solid probe of the respective residues following derivatization by methylation and trimethyl silylation. The confirmatory evidence was the appearance in the mass spectral data from residue fractions of molecular ions and/or fragments that were also present in the corresponding standards. The presence of zeatin riboside was not confirmed because a mass spectrum could not be successfully recorded. Mass spectral ions for the other five compounds of interest were: gibberellic acid (mol wt 346), 504(1 Me, 2 TMS), 238, 208; zeatin (mol wt 219), 291 (1 TMS), 276, 201, 188, 160; kinetin (mol wt 215), 301 (1Me, 1 TMS), 287 (1 TMS), 229 (1 Me); indole acetic acid (mol wt 175), 319 (2TMS), 261 (1Me, 1 TMS), 246 (1 TMS), 189 (1Me), 130; abscisic acid (mol wet 264), 278 (1Me) 264, 205, 190.

The HPLC analyses (Table 6) showed the gibberellic acid and abscisic acid were present in the highest concentrations in all tissues. The content of all compounds was higher in leaves than in leaf galls, and higher in stems than in stem galls. It has been shown that gall-producing plants may respond to some agent associated with an insect attack by eliciting the biosynthesis of plant growth regulators (Byers et al., 1976). Although the galls possessed chlorophyll when harvested, (so the galls were presumably of recent formation), this proposed increase was not evident in this test. Analysis at a still earlier stage may have confirmed this hypothesis. Also, other unidentified compounds may have contributed to gall formation.

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# EFFECTS OF SURFACTANTS, pH, AND CERTAIN CATIONS ON PRECIPITATION OF PROTEINS BY TANNINS

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**Abstract**—Tannic acid and pin oak tannins precipitate large amounts of the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), over a wide pH range (6.15–9.30) in the presence of sodium, potassium, magnesium, and calcium ions at concentrations comparable to those reported in the gut fluids of lepidopteran herbivores. The presence of lysolecithin, a surfactant known to be present in the gut fluids of some insects, significantly reduces the amount of RuBPC precipitated under these conditions. We conclude that high detergency is far more effective than high alkalinity in countering the potential protein-precipitating properties of tannins. We further conclude that tannins do not deserve the status they were once accorded as general, all-purpose, dose-dependent, antidigestive defensive chemicals. We also describe the application of the Schaffner-Weissman protein assay for studying the protein-precipitating capacity of plant extracts. This method is far superior to the one we have used in our earlier studies.

**Key Words**—Tannins, digestibility-reducing substances, surfactants, detergency, RuBPC, herbivory, chemical defense, allelochemics.

## INTRODUCTION

Tannins, which are water-soluble phenolic compounds that occur widely in vascular plants, have been accorded an important role in protecting plant tissues from herbivory (Feeny, 1976; Rhoades and Cates, 1976; Swain, 1979). The ingestion of tannins has been shown to interfere with normal growth and development in many foliage-feeding insects, although tannin-tolerant species are also well known (Bernays, 1981). Since tannins are known to be protein precip-



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itants (van Sumere et al, 1975; Hagerman and Butler, 1981; McManus et al, 1983), it has been proposed that they might reduce the nutritive value of plant tissues by forming indigestible complexes with foliar proteins or by precipitating and inactivating digestive enzymes in the digestive tract of an herbivore (Feeny, 1976; Rhoades and Cates, 1976). While it is well-documented that tannins can act as feeding deterrents and toxins to some insect herbivores, the idea that they are digestibility-reducing substances is currently being challenged (Bernays, 1981; Martin and Martin, 1984).

Interactions between tannins and proteins are strongly influenced by pH, ionic strength, detergents, and the concentrations of certain specific ions (Goldstein and Swain, 1965; Feeny, 1970; van Sumere et al., 1975; Hagerman and Butler, 1978, 1981; Berenbaum, 1980; Oh et al., 1980; Martin and Martin, 1983, 1984; McManus et al., 1983). It would seem possible that an insect herbivore might counter the potential of tannins to reduce the digestibility of dietary protein if it were to maintain conditions in its gut that were unfavorable for the formation of insoluble complexes between the major proteins and tannins present in the ingested foliage. Indeed, it has been proposed that high gut alkalinity is an antitannin adaptation in lepidopteran larvae (Feeny, 1970; Berenbaum, 1980), and that detergency is a widespread characteristic of insect gut fluids that would counter the potential antidigestive properties of tannins (Martin and Martin, 1984).

It was the goal of this study to determine the influence of sodium, potassium, magnesium, and calcium ions, pH, and lysolecithin on the precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) by tannic acid and pin oak extracts. Sodium, potassium, magnesium, and calcium were chosen for examination because they are the major cations present in foliage. Their effects were studied at concentrations similar to those reported in the gut fluids of two species of Lepidoptera, *Philosamia cynthia* and *Bombyx mori* (Giordana and Sacchi, 1978). Experiments were conducted over a pH range (6.15–9.30) that includes values typically recorded in the gut fluids of herbivorous insect species. The effect of lysolecithin was studied because this surfactant substance has been shown to be present in the midgut contents of *Pieris brassicae* (Turunen and Kastari, 1979). RuBPC was chosen as the test protein because it is a major dietary protein of any foliage-feeding insect, often making up as much as 25% of the total protein and 25–50% of the soluble protein in leaf tissue (Singer et al., 1952; Akazawa, 1970; Lyttleton, 1973; Jensen and Bahr, 1977). The study has been designed so that we may determine whether the conditions that prevail in the gut fluids of typical insect herbivores favor or disfavor the precipitation of RuBPC by tannins.

This paper also describes a procedure for determining the amount of protein in an insoluble protein–tannin complex that is far superior to the one we described earlier (Martin and Martin, 1982, 1983, 1984).

## METHODS AND MATERIALS

*Effects of Salts on Precipitation of RuBPC by Tannic Acid.* A stock solution of RuBPC (0.6–0.7 mg/ml) in buffer (0.05 M) containing either sodium chloride (3 mM) and potassium chloride (160 mM) or magnesium chloride (30 mM) and calcium chloride (20 mM) was prepared by combining suitable volumes of a solution of RuBPC in buffer and a solution of the appropriate pair of salts in water. Chlorides were used because of their high solubility and because chloride is a major anion in the gut fluids of herbivorous insects. Any insoluble material was removed by centrifugation (15,000*g*, 15 min, 24°C), and the protein content of this stock solution was measured using the procedure described below. Controls were run in which no salts were included in the stock solution. To 1.8 ml of the stock solution, agitated on a vortex mixer, was added 100 $\mu$l of a freshly prepared solution of tannic acid (1.0 mg/ml or 3.0 mg/ml). After 10 min, the mixture was centrifuged (30,000*g*, 15 min, 24°C), the supernatant solution was removed, and the pellet was rinsed very gently two times with buffer and drained. The precipitated tannic acid–RuBPC complex was redissolved by stirring for 30 min at room temperature with 0.75 ml of a 1% SDS solution in 0.05 M Tris, pH 7.5. The protein content of this solution was measured using the procedure described below.

The RuBPC preparation used in these experiments (Sigma R-2000, lot 98C-7140) was found by HPLC analysis to be only 65–70% pure. The contaminant(s) absorbed in 282 nm were presumed to be proteinaceous. This RuBPC preparation was freely soluble in the salt-free buffers and in the buffers containing sodium and potassium chloride at pHs 6.90, 7.55, and 8.30. The presence of magnesium and calcium chloride in these buffers caused about 5% of the protein to precipitate out of solution. It was not established whether it was RuBPC or a contaminant that was precipitated. The RuBPC preparation was somewhat less soluble at pHs 6.15 and 9.30. About 25% of the protein precipitated from these buffers when magnesium and calcium chloride were present. At pH 6.15, even in the salt-free buffer or the buffer–sodium chloride–potassium chloride mixture, 10–15% of the protein either precipitated or failed to dissolve. As a result of these solubility characteristics, protein content varied slightly from one incubation mixture to another (0.900–1.213 mg/1.8 ml). This variability did not obscure the clear patterns that emerged from the study.

The buffers used in these experiments were 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.15), *N*-2-acetamido-2-aminoethanesulfonic acid (ACES, pH 6.90), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.55), *tris*-(hydroxymethyl)aminomethane (Tris, pH 8.30), and cyclohexylaminoethanesulfonic acid (CHES, pH 9.30).

*Effect of Lysolecithin on Precipitation of RuBPC by Tannic Acid or Pin Oak Tannins in Presence of Salts.* To an aliquot (1.3 or 1.7 ml) of a stock so-

lution, prepared as above, containing RuBPC (0.6–0.9) mg/ml), sodium chloride, potassium chloride, magnesium chloride, and calcium chloride in buffer (0.05 M) was added 100 $\mu$l of a solution of lysolecithin in buffer at a concentration that resulted in a final lysolecithin concentration of 0.06%. At pH 6.15 and 7.55, $[Na^+]$ = 2.5 mM, $[K^+]$ = 131.0 mM, $[Mg^{2+}]$ = 25.0 mM, and $[Ca^{2+}]$ = 16.0 mM; at pH 6.90, 8.30 and 9.30, $[Na^+]$ = 3.0 mM, $[K^+]$ = 160 mM, $[Mg^{2+}]$ = 30.0 mM, and $[Ca^{2+}]$ = 20.0 mM. In the controls, 100 $\mu$l of buffer replaced the lysolecithin. After 5 min, either 100 $\mu$l of a freshly prepared solution of tannic acid (3.0 mg/ml) or 100 $\mu$l of a solution of tannins extracted from mature pin oak foliage (2.5 mg dry wt) was added. After 10 min, any precipitate present was collected by centrifugation, rinsed, drained, and redissolved in 1% SDS in 0.5 M Tris (ph 7.5) as described above. The protein content was measured using the procedure described below.

*Preparation of Foliage Extract.* Mature pin oak (*Quercus palustris*) foliage was lyophilized and ground on a Wiley mill (60-mesh). Forty milligrams of leaf powder was extracted twice for 8 min with 1.6 ml of boiling 50% (v/v) aqueous methanol. The extract was concentrated to dryness at reduced pressure, and the residue redissolved in 1.6 ml of water. Material that did not dissolve was removed by centrifugation.

*Protein Assay.* Protein content was measured using the method of Schaffner and Weismann (1973). An aliquot of the test solution, containing 20–130 $\mu$g of protein, was made up to a volume of 0.75 ml by the addition of a solution of 1% SDS in 0.05 M Tris, pH 7.5. Protein was precipitated by adding 0.15 ml of 90% trichloroacetic acid and vortexing the mixture. After 2–5 min, the precipitate was adsorbed on a nitrocellulose membrane (0.45 $\mu$m) by vacuum filtration. The tube was rinsed once with 1.2 ml of 6% trichloroacetic acid, and the rinse was poured through the filter apparatus. The adsorbed protein was then stained by immersing the filter disk in a 0.25% solution of Amido black 10B in methanol–acetic acid–water (50:10:40 vol %) for 10 min. Excess, unbound Amido black 10B was removed from the stained disk by rinsing for 30–45 sec in water, then treating for a total of 4 min in three changes of destaining solvent (methanol–acetic acid–water, 90:2:8 vol %), rinsing again with water for 2–3 min, and finally blotting dry with filter paper. Protein-bound dye was then eluted by shaking for 10–15 min in 3.0 ml of eluting solution (25 mM NaOH, 0.05 mM EDTA in 50 vol % aqueous methanol). Absorbance was determined at 630 nm, and converted to micrograms of protein by the use of a calibration curve constructed from dilutions of a stock solution of RuBPC.

## RESULTS

*Improved Procedure for Determining Amount of Protein Precipitated by Tannin Solution.* In earlier studies, Martin and Martin (1982, 1983, 1984) de-

termined the amount of protein precipitated by a tannin solution indirectly by measuring the amount of protein in solution before and after the addition of the tannin solution, using the dye-binding assay of Bradford (1976). It was not possible to measure the amount of protein precipitated directly because a detergent solution is required to redissolve the precipitated protein–tannin complex and detergents interfere with the Bradford assay. With the discovery that surfactants may play a major role in preventing the precipitation of proteins by tannins in insect guts (Martin and Martin, 1984), it became evident to us that a different assay would be required if we were to explore this effect and extend our studies to insects with gut fluids containing high concentrations of detergent substances. We have found that the method of Schaffner and Weissman (1973) circumvents the problems of the Bradford assay and can be used to measure the amount of protein present in a protein–tannin precipitate. In this procedure the insoluble protein–tannin complex is dissolved in a 1% SDS/Tris solution. Then protein is precipitated by the addition of trichloroacetic acid, adsorbed on a filter membrane, and stained with Amido black 10. This method is a significant improvement over the one we used earlier in the measurement of the protein-precipitating capacity of tannin solutions and plant extracts, and we urge its routine adoption.

*Effect of Alkali Metal Ions and Alkaline Earth Metal Ions on Precipitation of RuBPC by Tannic Acid.* The amount of RuBPC precipitated from solution by the addition of tannic acid is highly dependent upon pH and the presence of alkali metal ions or alkaline earth ions (Table 1). The concentrations of sodium (3 mM), potassium (160 mM), magnesium (30 mM), and calcium (20 mM) were selected to resemble the concentrations of these same cations in the midgut lumen contents of two herbivorous lepidopteran larvae, *Bombyx mori* and *Philosamia cynthia*. In these two species, Giordana and Sacchi (1978) have reported the following concentrations (mM): $Na^+$, 1.3 and 1.0; $K^+$, 149.5 and 196.8; $Mg^{2+}$, 27.4 and 8.6; and $Ca^{2+}$, 19.6 and 11.0 for *B. mori* and *P. cynthia*, respectively.

At pHs 6.15 and 6.90, the presence of sodium and potassium ions favors the precipitation of RuBPC by tannic acid. At pH 6.15, 100 $\mu$g of tannic acid precipitates 49% of the RuBPC present in a solution containing sodium and potassium chloride, but only 17% when these salts are absent (runs 1 and 2). At pH 6.90, 300 $\mu$g of tannic acid precipitates 84% of the RuBPC when sodium and potassium chloride are present, but only 10% of the RuBPC from the salt-free buffer (runs 4 and 5). At the more alkaline pHs, 7.55 and 8.30, however, very little RuBPC is precipitated (less than 5%) even by 300 $\mu$g of tannic acid (runs 7, 8, 10 and 11). This effect of pH is evident even at the lower pHs when the amount of RuBPC precipitated by a given amount of tannic acid is compared at pH 6.15 and 6.90 (runs 2 and 5). At pH 6.15, 100 $\mu$g of tannic acid precipitates 49% of the RuBPC from the salt-containing buffer, whereas at pH 6.90 this amount of tannic acid precipitates less than 5% of the RuBPC.

TABLE 1. AMOUNTS OF RuBPC PRECIPITATED FROM SALT SOLUTIONS AT VARIOUS pH'S BY ADDITION OF SOLUTION OF TANNIC ACID

| Run | pH | Cation conc. (mM) | | | | RuBPC in incubation mixture (mg) | RuBPC precipitated (mg)[a] | |
|---|---|---|---|---|---|---|---|---|
| | | $Na^+$ | $K^+$ | $Mg^{2+}$ | $Ca^{2+}$ | | By 100 μg TA | By 300 μg TA |
| 1 | 6.15 | 0.0 | 0.0 | 0.0 | 0.0 | 1.21 | 0.20 ± 0.004 (6) | 1.11 ± 0.014 (6) |
| 2 | | 3.0 | 160.0 | 0.0 | 0.0 | 1.07 | 0.52 ± 0.010 (6) | 0.99 ± 0.027 (6) |
| 3 | | 0.0 | 0.0 | 30.0 | 20.0 | 0.90 | 0.80 ± 0.008 (6) | ND[b] |
| 4 | 6.90 | 0.0 | 0.0 | 0.0 | 0.0 | 1.18 | 0.03 ± 0.002 (6) | 0.12 ± 0.002 (6) |
| 5 | | 3.0 | 160.0 | 0.0 | 0.0 | 1.20 | 0.06 ± 0.003 (3) | 1.01 ± 0.011 (6) |
| 6 | | 0.0 | 0.0 | 30.0 | 20.0 | 1.13 | 0.85 ± 0.013 (6) | 1.02 ± 0.008 (6) |
| 7 | 7.55 | 0.0 | 0.0 | 0.0 | 0.0 | 1.18 | 0.02 ± 0.001 (6) | 0.02 ± 0.001 (6) |
| 8 | | 3.0 | 160.0 | 0.0 | 0.0 | 1.15 | 0.02 ± 0.001 (6) | 0.05 ± 0.002 (6) |
| 9 | | 0.0 | 0.0 | 30.0 | 20.0 | 1.09 | 0.32 ± 0.012 (6) | 0.61 ± 0.007 (7) |
| 10 | 8.30 | 0.0 | 0.0 | 0.0 | 0.0 | 1.18 | ND | 0.01 ± 0.001 (6) |
| 11 | | 3.0 | 160.0 | 0.0 | 0.0 | 1.18 | ND | 0.01 ± 0.001 (6) |
| 12 | | 0.0 | 0.0 | 30.0 | 20.0 | 1.13 | 0.31 ± 0.012 (6) | 0.60 ± 0.009 (6) |
| 13 | 9.30 | 0.0 | 0.0 | 30.0 | 20.0 | 0.92 | 0.38 ± 0.009 (6) | 0.65 ± 0.009 (5) |

[a] Values are the mean ± standard error, with the number of replicates given in the parentheses.
[b] ND, not determined.

The alkaline earth cations, magnesium and calcium, are even more effective than the alkali metal cations, sodium and potassium, at bringing about the precipitation of RuBPC by tannic acid. At pH 6.15 or 6.90, 100 μg of tannic acid precipitates 89% and 75% of the RuBPC from buffers containing magnesium and calcium chloride, respectively, but only 49% and 5% from the buffers containing sodium and potassium chloride (runs 2, 3, 5, and 6). Increased alkalinity does not nullify the effect of the alkaline earth ions in the same way it counters the effect of the alkali metal ions. Even at pHs 7.55, 8.30, and 9.30, significant quantities of RuBPC are precipitated from solutions containing magnesium and calcium chloride when tannic acid is added (runs 9, 12, and 13).

*Effect of Lysolecithin on Precipitation of RuBPC from Salt Solutions by Tannic Acid and Pin Oak Foliage Extracts.* Lysolecithin, a surfactant that has been detected in the gut fluids of *Pieris brassicae* (Turunen and Kastari, 1979), significantly reduces the amount of RuBPC precipitated by tannic acid (Table 2). Experiments were run in buffers containing all four cations at concentrations comparable to those reported in the gut fluids of *B. mori* and *P. cynthia*. The lysolecithin concentration (0.06%) was about 15 times the critical micelle concentration (CMC), which is the concentration at which there is a transition between the surfactant in the free, unassociated state and the micellar state. Sur-

TABLE 2. EFFECT OF LYSOLECITHIN ON AMOUNT OF RuBPC PRECIPITATED FROM SALT SOLUTIONS AT VARIOUS pH'S BY ADDITION OF TANNIC ACID SOLUTION OR EXTRACT OF PIN OAK FOLIAGE

| pH | Lysolecithin conc. (%) | RuBPC in incubation mixture (mg) | RuBPC precipitated (mg)[a] | |
|---|---|---|---|---|
| | | | By 300 μg TA | By foliage extract[b] |
| 6.15[c] | 0.00 | 0.96 | 0.82 ± 0.004 (4) | 0.77 ± 0.009 (6) |
| | 0.06 | 0.96 | 0.04 ± 0.001 (5) | 0.03 ± 0.000 (6) |
| 6.90[d] | 0.00 | 1.18 | 1.09 ± 0.004 (5) | ND[e] |
| | 0.06 | 1.18 | 0.05 ± 0.001 (6) | ND |
| 7.55[c] | 0.00 | 1.13 | 0.66 ± 0.019 (6) | 0.51 ± 0.013 (5) |
| | 0.06 | 1.13 | 0.15 ± 0.004 (6) | 0.08 ± 0.004 (5) |
| 8.30[d] | 0.00 | 1.13 | 0.55 ± 0.016 (6) | ND |
| | 0.06 | 1.13 | 0.18 ± 0.004 (6) | ND |
| 9.30[d] | 0.00 | 0.96 | 0.51 ± 0.006 (5) | ND |
| | 0.06 | 0.96 | 0.21 ± 0.002 (6) | ND |

[a]Values are the mean ± standard error, with the number of replicates given in the parenthesis.
[b]From 2.5 mg (dry wt) milled leaf material.
[c]$[Na^+]$ = 2.5 mM, $[K^+]$ = 131.0 mM, $[Mg^{2+}]$ = 25.0 mM, $[Ca^{2+}]$ = 16.0 mM.
[d]$[Na^+]$ = 3.0 mM, $[K^+]$ = 160.0 mM, $[Mg^{2+}]$ = 30.0 mM, $[Ca^{2+}]$ = 20.0 mM.
[e]ND, not determined.

face-tension measurements (Martin and Martin, 1984) have shown that in the gut fluids of *Manduca sexta* (on artificial diet), *Malacosoma sp.* (on black cherry foliate), and *Colias philodice* (on alfalfa foliage), surfactants are present at concentrations of 10, 20, and 30 times CMC, respectively. At pHs 6.15, 6.90, and 7.55, lysolecithin virtually prevents the precipitation of RuBPC by tannic acid. At pHs 8.30 and 9.30, lysolecithin reduces the amount of RuBPC precipitated from 49% to 16% and from 54% to 21%, respectively.

In earlier papers, Martin and Martin (1982, 1983) demonstrated that extracts of mature pin oak foliage have high protein-precipitating capacity, presumably due to the presence of high concentrations of tannins. In this study we demonstrate that at pHs 6.15 and 7.55, lysolecithin is also effective at preventing pin oak tannins from precipitating RuBPC from a solution containing alkali metal and alkaline earth cations (Table 2).

## DISCUSSION

The objective of this study was to ascertain whether the conditions that prevail in the gut fluids of insect herbivores favor or disfavor the formation of insoluble complexes between RuBPC, the major protein of photosynthetic tissue, and plant tannins. Our results clearly indicate that conditions are unfavorable for the formation of such complexes. Our findings, therefore, are not supportive of the hypothesis that tannins reduce the digestibility of plant tissues by forming insoluble, indigestible complexes with ingested protein in an herbivore's gut.

Since potassium, magnesium, and calcium are the major cations in plant tissue, it is to be expected that they would also be the major cations in the midgut fluids of an insect herbivore. Potassium ions are also pumped into the midgut from the hemolymph. These ions favor the formation of insoluble RuBPC–tannin complexes. However, other characteristics of the gut fluid effectively counter the protein-precipitating potential of tannins, even in the presence of potassium, magnesium, and calcium ions.

Previous investigators have suggested that an elevated midgut pH may be an antitannin adaptation. Our results suggest that high detergency is far more effective than high alkalinity in reducing the amount of protein precipitated by tannins. While an increase in pH can virtually prevent the precipitation of RuBPC by tannins when only sodium and potassium ions are present, the amount precipitated is only moderately reduced by an increase in pH when magnesium and calcium ions are present. By contrast, lysolecithin significantly reduces the amount of protein precipitated when all four ions are present and is effective over a wide pH range.

It is becoming increasingly evident that the digestive systems of insects possess several characteristics that counteract the potential antidigestive properties of tannins (Bernays, 1981). This study and our earlier one (Martin and

Martin, 1984) suggest that the presence of surfactants in the gut fluid may be one of the most effective and widespread of these traits. Indeed, it is clear that tannins are not the general, all-purpose, dose-dependent, antidigestive, defensive chemicals they were once thought to be.

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# OLFACTORY BEHAVIOR OF RED FLOUR BEETLE *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae) TOWARDS NATURAL FATTY ACID ESTERS

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**Abstract**—The olfactory responses of red flour beetles, *Tribolium castaneum* (Herbst), to methyl and ethyl ester of $C_{14}$-$C_{18}$ fatty acid prepared from tallow were studied. Methyl octadecanoate and ethyl octadecanoate discouraged aggregation of beetle adults. Ethyl 9.12-octadecadienoate acted as a repellent. Methyl pentadecanoate and ethyl tetradecanoate induced copulation at the level of $5 \times 10^2$ and $2 \times 10^3$ $\mu$g, respectively. The maximum response was observed at the level of $5 \times 10^2$ $\mu$g of the same compounds, and these compounds appear to stimulate males only.

**Key Words**—*Tribolium castaneum*, Coleoptera, Tenebrionidae, red flour beetle, olfactory behavior, ethyl 9,12-octadecadienoate, methyl octadecanoate, methyl tetradecanoate, methyl pentadecanoate, ethyl tetradecanoate, ethyl pentadecanoate, ethyl octadecanoate.

## INTRODUCTION

The red flour beetle *Tribolium castaneum* (Herbst) is a common pest of various foodstuffs, particularly cereals and their products (Sokoloff, 1972). The behavior of an insect to an attractant component is an important stimulus-response parameter in olfactory physiology (Kaissling, 1971), and applied entomology (Mankin et al., 1980). The ultimate aim of these studies is the design of effective pest management for *T. castaneum*. Some investigations were carried out on the chemosensory effect (Starrat and Loschiavo, 1972; House and Graham, 1967; Cohen et al., 1974) of lipids, particularly fatty acids, on *T. castaneum*. Suzuki (1980) identified the molecular structure of the aggregation pheromone of *T. castaneum* as 4,8-dimethyldecanal; recently Levinson and Mori (1983) assigned



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the stereochemistry as (4*R*,8*R*)-(−) and determined that it acts as a sex attractant for the female and an aggregation pheromone for the male of this species. In the present investigation, esters of seven fatty acids prepared from tallow were tested on *T. castaneum* to determine the behavioral responses. These esters are the intermediates in the preparation of the aldehyde, the natural pheromone of *T. castaneum*.

## METHODS AND MATERIALS

*Insects.* The culture of beetles was maintained in darkness in 1-gal jars, one-third filled with a mixture of wheat flour, cornmeal, and oatmeal at 30 ± 1°C and 70% relative humidity. Pupae were removed daily from the cultures and sex segregated (Halstead, 1963). The emerging adults were removed and bioassayed.

*Olfactory Response Bioassay.* Methyl and ethyl ester of $C_{14}$–$C_{18}$ fatty acids were dissolved in hexane (1000 μg/ml) and applied to 24-cm filter paper disks (Whatman No. 1) in amounts ranging from $2 \times 10^1$ to $2 \times 10^3$ μg. The assays were performed at 30 ± 10°C by a two-choice technique designed by Loschiavo (1965a) and described by Levinson and Bar Ilan (1967). The response of insects to the compounds was achieved by modifying the test suggested by Cohen et al., (1974). In the design called "devised," the filter paper circles were placed inside the Petri dish. One hundred beetles (50 males and 50 females) were transferred to the experimental Petri dishes and observation began immediately. Each group was observed for 1 min, and the number of copulations and insect aggregations were noted. The observations were repeated at intervals of 5 min over a 30-min period. The response at the end of the 30-min period was estimated using the formula given by Levinson and Bar Ilan (1967).

To determine the response of males and females to bioassay, the chemicals were introduced by applying 2.5 ml of different concentrations separately to Petri dishes of filter paper of 8 mm in diameter, and then the Petri dishes were covered. The filter paper in the control Petri dish was either left dry or treated with solvent. The males and females were released in the Petri dishes separately and observation began immediately. The number of males mounting other males and the number of adult males and females aggregated in treated area and control were recorded.

An analysis of variance was used to analyze data, and only differences significant at the 1% level are considered statistically significant.

*Compounds Tested.* The following methyl and ethyl ester of $C_{14}$–$C_{18}$ acids prepared from tallow were employed for assay: methyl octadecanoate, methyl tetradecanoate, methyl pentadecanoate, ethyl octadecanoate, ethyl tetradecanoate, ethyl pentadecanoate, and ethyl 9,12-octadecadienoate. All the esters were pure and showed a single spot in thin-layer chromatography.

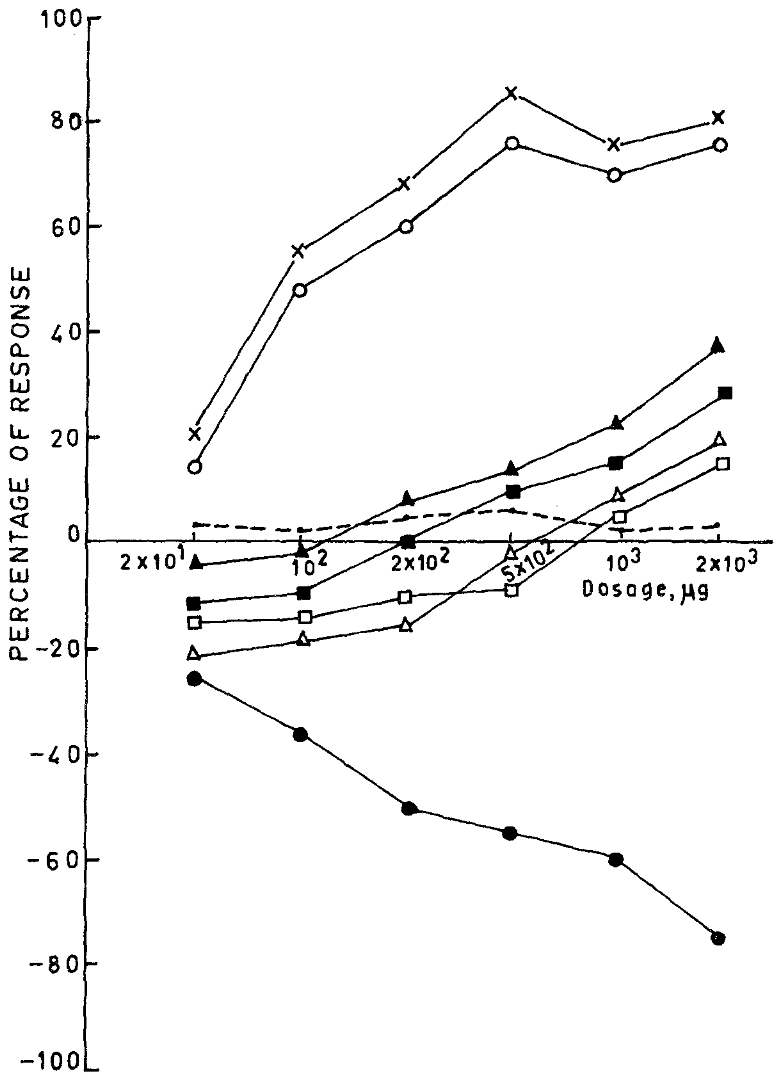


FIG. 1. Olfactory response of *Tribolium castaneum* (Herbst) to methyl and ethyl ester of fatty acids prepared from tallow.

## RESULTS

The results summarized in Figure 1 reveal the responsiveness to methyl and etheyl esters of natural $C_{14}$–$C_{18}$ fatty acids. Ethyl 9,12-octadecadienoate strongly repelled the insects. Methyl octadecanoate and its ethyl analog discouraged aggregation. The statistical test comparing each of the experimental compounds and the control indicates that no significant difference between ethyl pentadecanoate, methyl octadecanoate, methyl tetradecanoate, and control was recorded. A highly significant difference was recorded in the control, methyl pentadecanoate, and ethyl tetradecanoate. Further statistical analysis indicates that no significant difference was observed in methyl pentadecanoate and ethyl tetradecanoate in any tests. They apparently have the same effect, although not to the same degree. Methyl pentadecanoate exhibited maximal arrestant activity at the level of $5 \times 10^2$ $\mu$g. Ethyl tetradecanoate displayed the same effect in encouraging a high degree of attraction at the same level.

The results presented in Table 1 indicate that levels of either $2 \times 10^3$ $\mu$g of ethyl tetradecanoate, $5 \times 10^2$ $\mu$g of methyl pentadecanoate or $2 \times 10^3$ $\mu$g of methyl tetradecanoate induced copulation in 80% of insects.

It is obvious from Table 2 that males mounted other males very frequently and showed a high degree of aggregation in areas treated with methyl pentadecanoate at the level of $5 \times 10^2$ $\mu$g. No significant difference was recorded with this compound and ethyl tetradecanoate at the same level. Moreover, in case of females, moderate aggregation was recorded at different concentrations of methyl pentadecanoate and ethyl tetradecanoate. Thus, these substances appear to function as male sex stimulants. Methyl octadecanoate and ethyl penta-

TABLE 1. EFFICIENCY OF NATURAL FATTY ACID ESTERS PREPARED FROM TALLOW FOR MATING BEHAVIOR IN MALE *Tribolium castaneum*

| Compound | Amount ($\mu$g) of esters inducing copulation (%) | | | |
|---|---|---|---|---|
| | 20 | 50 | 80 | Control |
| Methyl octadecanoate | $5 \times 10^2$ (0.5 mg) | | ++[a] | + |
| Methyl tetradecanoate | $5 \times 10^2$ (0.5 mg) | | $2 \times 10^3$ (2 mg) | – |
| Methyl pentadecanoate | | $5 \times 10^1$ (0.05 mg) | $5 \times 10^2$ (0.5 mg) | – |
| Ethyl octadecanoate | $5 \times 10^2$ (0.5 mg) | | – | + |
| Ethyl tetradecanoate | | $10^2$ (0.1 mg) | $2 \times 10^3$ (2 mg) | – |
| Ethyl pentadecanoate | $5 \times 10^2$ (0.5 mg) | | ++ | + |
| Ethyl 9,12-octadecanoate | – | | – | + |

[a]+, copulation in below 10% beetles; ++, $10^3$ $\mu$g failed to induce mating in 80% of the beetles; –, no concentration induced copulation.

TABLE 2. MEAN NUMBER OF MALE MOUNTING PAIRS AND FEMALE ADULTS OF RED FLOUR BEETLE IN TREATED AREA AT DIFFERENT CONCENTRATIONS ($\mu g$) OF FATTY ACID ESTERS

| | Mounting pairs of male in each treatment[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Compound | Control | $2 \times 10^1$ | $10^2$ | $2 \times 10^2$ | $5 \times 10^2$ | $10^3$ | $2 \times 10^3$ |
| *Males*[b] | | | | | | | |
| Methyl octadecanoate | $0_a$ (5) | $0_a$ (0) | $0_a$ (3) | $0_a$ (3) | $1_b$ (10) | $3_b$ (9) | $3_b$ (13) |
| Methyl tetradecanoate | $0_a$[c] (7) | $0_a$ (3) | $0_a$ (3) | $2_b$ (7) | $2_b$ (7) | $1_b$ (7) | $1_b$ (11) |
| Methyl pentadecanoate | $0_a$ (5) | $2_{bc}$ (18) | $2_{bb}$ (21) | $7_{bb}$ (23) | $15_c$ (18) | $5_b$ (15) | $7_b$ (19) |
| Ethyl octadecanoate | $0_a$ (9) | 0 (0) | $0_a$ (5) | $0_a$ (3) | 0 (0) | $1_a$ (5) | $1_a$ (7) |
| Ethyl tetradecanoate | $0_a$ (5) | $3_b$ (15) | $5_{bc}$ (23) | $5_{bb}$ (21) | $13_c$ (17) | $3_b$ (11) | $3_b$ (17) |
| Ethyl pentadecanoate | $0_a$ (3) | 0 (0) | 0 (0) | $2_b$ (11) | 0 (0) | 0 (0) | 0 (0) |
| Ethyl 9,12-octadienoate | $0_a$ (7) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Females*[a] | | | | | | | |
| Methyl octadecanoate | $0_a$ (21) | 0 (0) | 0 (0) | 0 (0) | $0_a$ (3) | $0_a$ (2) | $0_a$ (2) |
| Methyl tetradecanoate | $0_a$ (5) | 0 (0) | 0 (0) | $0_a$ (7) | $0_a$ (8) | $0_{ab}$ (9) | $0_b$ (11) |
| Methyl pentadecanoate | $0_a$ (5) | $0_a$ (8) | $0_a$ (8) | $0_a$ (8) | $0_a$ (7) | $0_a$ (3) | $0_a$ (3) |
| Ethyl octadecanoate | $0_a$ (3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | $0_a$ (2) |
| Ethyl tetradecanoate | $0_a$ (3) | $0_a$ (3) | $0_a$ (3) | $0_{ab}$ (8) | $0_{ab}$ (9) | $0_a$ (3) | $0_a$ (3) |
| Ethyl pentadecanoate | $0_a$ (7) | 0 (0) | 0 (0) | 0 (0) | $0_a$ (4) | $0_a$ (3) | $0_a$ (3) |
| Ethyl 9,12-octadienoate | $0_a$ (7) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

[a] Means in each row and column followed by the same letter are not significantly different at 1% level with Duncan's multiple-range test.
[b] Comparisons of means are made for single sex.
[c] Figures in parenthesis denote the number of adults entered in the treated area.

decanoate discouraged aggregation in male and female *T. castaneum* at all levels tested.

## DISCUSSION

From the foregoing results, it is evident that methyl pentadecanoate induced intense aggregation and acted as a sex attactant towards males. Moreover, ethyl tetradecanoate was found to induce successful aggregation and copulation in *T. castaneum* at high concentrations. A previous report (Cohen et al., 1974) revealed that aggregation in *T. castaneum* was induced by tridecanoic, pentadecanoic, palmitic, stearic, and oleic acids. Ethyl 9,12-octadecadienoate acted as a repellent. Methyl tetradecanoate and ethyl pentadecanoate were not effective in inducing aggregation. Methyl octadecanoate and ethyl octadecanoate discourage aggregation as well as copulation in *T. castaneum.*

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# STRUCTURE-ACTIVITY RELATIONSHIPS AMONG MAYTANSINOIDS IN THEIR EFFECT ON THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (HÜBNER)[1]

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**Abstract**—Five maytansinoids from *Maytenus* (Celastraceae) and *Putterlickia* (Rhamnaceae) species were tested for biological activity against the European corn borer *Ostrinia nubilalis*. Maytanbutine, maytansine, and maytanvaline, all of which contain an amino acid residue at C-3, were active and comparable in their effect on larvae to trewiasine, a known active, amino acid-containing maytansinoid from *Trewia nudiflora*. Maytanacine, which has an acetate group at C-3, was not as active as maytansine, maytanvaline, maytanbutine, or trewiasine, but significantly retarded the development of the larvae. Normaysine, which has no oxygen substituent at C-3, had no significant effect on mortality and only moderate effect on development of the larvae. The presence of the amino acid moiety at C-3 appears to be an important factor for the biological activity of maytansinoids.

**Key Words**—Maytansinoids, maytanacine, maytanbutine, maytanvaline, normaysine, trewiasine, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, insect toxicity, antifeedants, pupation inhibitors.

## INTRODUCTION

Recently, Powell et al. (1981, 1982) isolated a series of biologically active maytansinoids from ethanolic extracts of *Trewia nudiflora* L. (Euphorbiaceae), of which trewiasine is the most abundant. These compounds were unique in that

[1] Lepidoptera: Pyralidae.
[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

they contained a 15-methoxyl group, thus far found only in maytansinoids from *Trewia.* Moreover, some of these (treflorine, trenudine, and *N*-methyl trenudone) (Structure 1) also contained two fused macrocyclic rings (Powell et al., 1982). In addition to the 19-membered ring characteristic of all previously known maytansinoids, these new compounds have a 12-membered ring joining C-3 and the amide nitrogen at C-18. We conducted bioassays and reported that these compounds from *Trewia* caused mortality to larvae of the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Freedman et al., 1982), per-

Treflorine R = H

Trenudine R = OH

*N*-Methyltrenudone

STRUCTURE 1.

haps because of antifeedant action. We also observed that trewiasine was highly toxic and showed antifeedant action toward both the adult striped cucumber beetle *Acalymma vittatum* (F.) and the larvae of the codling moth, *Cydia pomonella* (L.) (Reed et al., 1983). The present study was undertaken to determine whether a wide range of maytanside esters having other structural features also showed similar activity. In the present study, we report results of the bioassay against *O. nubilalis* for five previously known maytansinoids from plant extracts of the Celastraceae (Kupchan et al., 1975, 1977) and Rhamnaceae (Wani et al., 1973).

## METHODS AND MATERIALS

Samples of maytanacine, maytanbutine, maytansine, maytanvaline, and normaysine (Figure 1) were originally obtained from ethanolic extracts of seed of *Maytenus* (Celastraceae) or *Putterlickia* (Rhamnaceae) species and were furnished by Dr. Albert T. Sneden (Department of Chemistry, Virginia Commonwealth University, Richmond, Virginia 23284). Trewiasine, used as a positive control, was obtained from *T. nudiflora* (Powell et al., 1981) and was furnished by R.G. Powell. Egg masses of *O. nubilalis* were supplied by W.D. Guthrie (Corn Insects Research Laboratory, ARS, USDA, Ankeny, Iowa 50021). Larvae were reared and tested as described by Freedman et al. (1979) and summarized in Table 1. Mortality was determined after 11 days only.

## RESULTS AND DISCUSSION

Table 1 shows the results of testing all six maytansinoid compounds, each at three dose levels. The data indicate that the activity of maytanbutine, maytansine, and maytanvaline is comparable to that of trewiasine. Upon being exposed to diet containing the active compounds, *O. nubilalis* larvae crawled away from the diet as though they were avoiding it. We reported previously that *O. nubilalis* larvae behave this way on diets treated with trewiasine and other maytansinoids from *Trewia* (Freedman et al., 1982). Dead or alive, the larvae were invariably much smaller in size than the controls, as if they had eaten little or nothing.

Maytanacine and normaysine caused some mortality of larvae, although they were less effective than the compounds discussed in the preceding paragraph. However, none of the larvae exposed to maytanacine pupated. In contrast, a considerable proportion of the larvae exposed to normaysine did pupate, although this proportion (42%) was significantly less than in the controls. [A $\chi^2$ test indicates significantly higher pupation ($P < 0.01$) for controls.]

There seems to be a correlation between structure and biological activity of these compounds. All contain the unique maytansinoid ring system, and in

Maytanacine

| | | |
|---|---|---|
| Maytanbutine | $R^1 = CH(CH_3)_2$ | $R^2 = H$ |
| Maytansine | $R^1 = CH_3$ | $R^2 = H$ |
| Maytanvaline | $R^1 = CH_2CH(CH_3)_2$ | $R^2 = H$ |
| Trewiasine | $R^1 = CH(CH_3)_2$ | $R^2 = OCH_3$ |

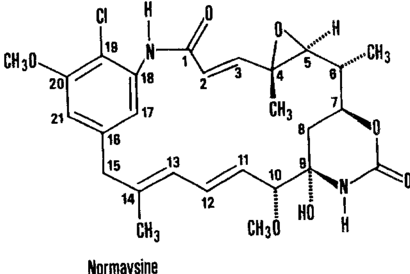


FIG. 1. Maytansinoids tested against the European corn borer *Ostrinia nubilalis*.

TABLE 1. MORTALITY OF *Ostrinia nubilalis* LARVAE TREATED WITH SELECTED MAYTANSINOIDS

| Compounds | Dose (mg)[a] | Mortality (%) (11 Days)[b] | Number alive (11 Days) | Pupation (%) (17 Days)[c] | $LD_{50}$ (mg/cup)[d] | $LD_{50}$ (ppm) | 95% Confidence interval (ppm) |
|---|---|---|---|---|---|---|---|
| Maytanacine | 0.1 | 17 | 14 | 0 | –[e] | – | – |
| | 0.05 | 6 | 15 | 0 | | | |
| | 0.0125 | 16 | 11 | 0 | | | |
| Maytanbutine | 0.2 | 68*[f] | 6 | 0 | 0.085 | 21 | 9–58 |
| | 0.05 | 43* | 8 | 0 | | | |
| | 0.0125 | 9 | 12 | 0 | | | |
| Maytansine | 0.2 | 78* | 4 | 0 | 0.090 | 22 | 10–54 |
| | 0.05 | 18 | 13 | 0 | | | |
| | 0.0125 | 22 | 8 | 0 | | | |
| Maytanvaline | 0.2 | 78* | 4 | 0 | 0.084 | 21 | 10–47 |
| | 0.05 | 25 | 12 | 0 | | | |
| | 0.0125 | 16 | 11 | 0 | | | |
| Normaysine | 0.2 | 0 | 18 | 33.3 | – | – | – |
| | 0.05 | 11 | 15 | 60.0 | | | |
| | 0.0125 | 0 | 19 | 36.8 | | | |
| Trewiasine | 0.2 | 72* | 5 | 0 | 0.074 | 18 | 7–46 |
| | 0.05 | 47* | 6 | 0 | | | |
| | 0.0125 | 10 | 11 | 0 | | | |
| All controls | 0 | 6 | 105 | 68.6 | – | – | – |

[a]Each dose was mixed with 4 g diet to which five 7-day-old larvae (third instar) were added. This was replicated four times. Twenty larvae were used per dose and 120 larvae as controls. During the 11-day observation period, larvae were missing from some replicates and were assumed to have been cannibalized; these larvae were not counted. Analyses of data were based only on larvae observed as dead or alive. At the highest dose, 5% of the larvae were missing, but 28% were missing at the lowest dose.

[b]Data adjusted for control mortality by Abbot's formula: % mortality = $(x - y)\ 100/x$ where $x$ is the % of insects living in the control and $y$ is the % living in the treated samples (Abbot, 1925). Mortality in controls after 11 days was 6%. With 20 or fewer insects (not counting missing larvae) per dose, sampling variability accounts for the apparent contradictions between % mortality at different dose levels.

[c]% Pupation is the percentage of insects alive at 11 days that had reached the pupa or adult state at 17 days.

[d]Estimates of $LD_{50}$ were made using the standard probit analysis of Daum (1970).

[e]$LD_{50}$ not calculated for materials that caused little mortality.

[f]*Significantly different from control at $P = 0.05$.

all of the more active members of the series, the hydroxyl at C-3 is esterified with an amino acid. A methoxyl group at C-15 seems to make little difference. Maytanacine, which is not lethal to larvae but acts as a pupation inhibitor, is a maytanside ester with acetate at C-3 instead of an amino acid residue (Kupchan et al., 1975). Normaysine, which seemed to have no effect on the larvae, has no oxygen function at C-3, but instead has a double bond between C-2 and C-3 (Kupchan et al., 1977). Our observations parallel those of Kupchan et al. (1978), who showed that maytansinoids which lack the C-3 ester show no antileukemic (PS) activity. Thus it appears that the insect-controlling activity of the maytansinoids also can be predicted from the structure, but this requires further study.

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# EFFECT OF HYPOXANTHINE-3(*N*)-OXIDE AND HYPOXANTHINE-1(*N*)-OXIDE ON CENTRAL NERVOUS EXCITATION OF THE BLACK TETRA *Gymnocorymbus ternetzi* (CHARACIDAE, OSTARIOPHYSI, PISCES) INDICATED BY DORSAL LIGHT RESPONSE

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**Abstract**—The change of state in the central nervous system of *Gymnocorymbus ternetzi* after detection of hypoxanthine-1(*N*)-oxide, hypoxanthine-3(*N*)-oxide, and of the alarm substance from conspecifics was measured quantitatively by means of the fishes' equilibrium behavior. The fish swam freely in a tiny cage, illuminated horizontally from one side. The change of the angle of inclination of the dorsoventral axis of the fish was registered by means of a videorecorder. The recordings were later measured on the monitor in single frames at 0.2-sec intervals where the equilibrium position of the fish could be accurately determined $\pm 1°$. Various substances were presented to the fish, and their effects upon equilibrium position were recorded. An enhanced optical alertness shown by an increase in the fishes' inclination was generally produced with alarm substance. Without any additional stimulation, the factor $U$, representing quantitatively the degree of the change of central state, varied slightly within the experimental period of 1 min; however, this factor never exceeded $U = 1.0 \pm 0.15$ in control fish. The increase of $U$ usually exceeded considerably the value 1.15 when skin extract from conspecifics or 7–8 μg of hypoxanthine-3(*N*)-oxide were given. However, when hypoxanthine-1(*N*)-oxide was presented, $U$ generally did not exceed 1.15. The difference between hypoxanthine-3(*N*)-oxide and hypoxanthine-1(*N*)-oxide was highly significant. This result is in accordance with the findings on fish schools of *Danio malabaricus*, where hypoxanthine-3(*N*)-oxide elicited the fright reaction, but hypoxanthine-1(*N*)-oxide was ineffective. The results support the hypothesis that the alarm substance from the skin of *Phoxinus phoxinus* is identical with hypoxanthine-3(*N*)-oxide. The results with alarm substance or hypoxanthine-3(*N*)-oxide did not show any adaptation. This was also true in fish that were stimulated repeatedly at intervals of a couple of minutes only. In



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*Gymnocorymbus,* which has compensated for removal of the otolith of one utriculus, conspecific skin extract triggers the typical postoperative phenomenon, i.e., rotation around the fishes' long axis towards the operated side. Whereas such a decompensation could be elicited by hypoxanthine-3(*N*)-oxide as well, hypoxanthine-1(*N*)-oxide had no effect. This finding is interpreted as an effect of the alarm substance and of hypoxanthine-3(*N*)-oxide on the centers of equilibrium.

**Key Words**—Fish, *Gymnocorymbus ternetzi* (Boulenger), fright reaction, pheromone, alarm substance, hypoxanthine-3(*N*)-oxide, hypoxanthine-1(*N*)-oxide, dorsal light response, equilibrium behavior, central nervous excitation.

## INTRODUCTION

Most Ostariophysi show a characteristic fright reaction when they detect the alarm substance derived from injured skin of conspecifics or other ostariophysean species. The fright reaction changes the behavior drastically. The fish seem terrified; they swim away in confusion and hide. They not only flee, but their vigilance increases. The fright reaction, first discovered by Karl von Frisch (1941), has been reviewed in detail (Pfeiffer, 1962, 1963, 1966, 1974, 1977, 1982; Smith, 1982). It was suggested that the alarm substance might cause a change of central nervous excitation ["*Umstimmung*"sensu von Holst (1950a–c) or "change of central state"], and this might be measured by means of dorsal light response (Pfeiffer, 1964), particularly since the optical alertness is enhanced in frightened fish. Pfeiffer and Riegelbauer (1978) showed that the change of state, activated by the detection of alarm substance, can be quantitatively recorded by means of equilibrium orientation.

In contrast to the results obtained with xanthopterin and 7-acetonylxanthopterin, skin extract containing alarm substance or isolated pure genuine alarm substance, as well as isoxanthopterin and 6-acetonylisoxanthopterin, yield a clear change of state. Only those substances which arouse the fright reaction in behavior experiments with fish schools are effective in experiments on the dorsal light response (Pfeiffer and Riegelbauer, 1978; Pfeiffer, 1978). However, isoxanthopterin and 6-acetonylisoxanthopterin are not identical with the genuine alarm substance (Pfeiffer, 1975).

The goal of this investigation was to compare the effects of hypoxanthine-1(*N*)-oxide and hypoxanthine-3(*N*)-oxide with the effect of the alarm substance, since it has been indicated that a relationship exists between the genuine alarm substance and hypoxanthine-3(*N*)-oxide (Argentini, 1976). Secondly, we wished to test whether $CaCl_2$ and/or KCl increase the effect of hypoxanthine-3(*N*)-oxide, since both are present in fish skin and, indeed, are difficult to separate from genuine alarm substance (Argentini, 1976). To add to this, $CaCl_2$ proves to be a highly effective odor stimulant to fish (Bodznick, 1978). Finally we studied

the effect of removing the otolith of one utriculus on the excitation elicited by alarm substance or hypoxanthine-3(*N*)-oxide.

## METHODS AND MATERIALS

The black tetra, *Gymnocorymbus ternetzi* (Boulenger 1895), served as the experimental fish; this species proved excellent in earlier investigations (Pfeiffer and Riegelbauer, 1978). The black tetra has a marked fright reaction, and its equilibrium orientation is well known. Von Holst (1950a, b) showed that physiological states may influence the equilibrium orientation profoundly and that the effects may be measured quantitatively by means of the equilibrium position of fish. If a black tetra is illuminated horizontally, it inclines its dorsoventral axis and turns its dorsal side towards the light.

The equilibrium position of the fish is determined using static and optic stimuli and the evaluation of their effects on the central nervous system. The static component ($D_{st}$) and the optic component ($D_{opt}$) are opposite to each other: $D_{st} = -D_{opt}$ . $D_{st} = cF \sin \alpha$; $c$ = proportionality constant, $F$ = gravity, $\alpha$ = angle between the dorsoventral axis of the fish and the perpendicular line, i.e., the inclination of the fish (Figure 1C). $D_{opt} = f(L) \sin \beta$; $f(L)$ = dependence of the optic turning tendency on the light intensity, $\beta$ = angle between dorsoventral axis of fish and light incidence (Figure 1C).

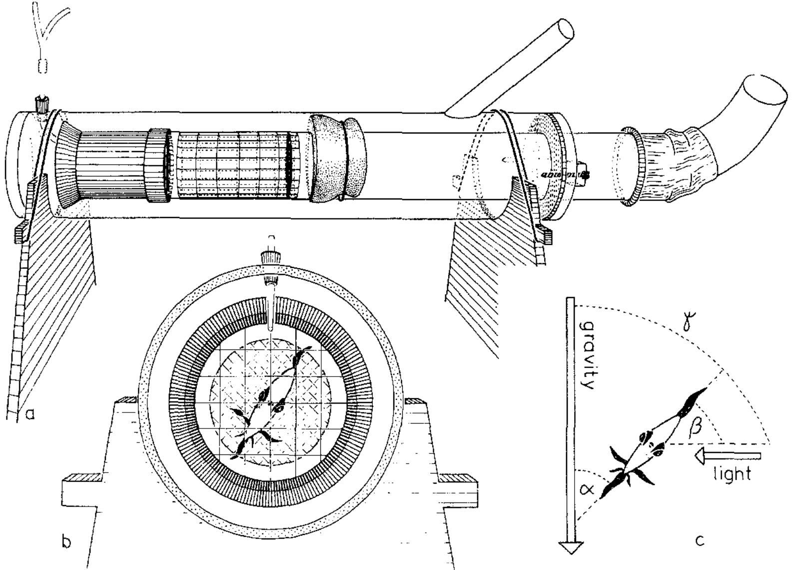


FIG. 1. Semischematic diagram showing a part of the apparatus. (a) lateral view; (b) frontal view; and (c) denotation of the angles. Explanation in the text.

The evaluation of the optic and static components changes; the ratio sin $\beta$ to sin $\alpha$ depends both on additional external stimuli and on the central nervous excitation ("central state") of the fish. It may cause a heightened receptivity for the optic stimulus, which is shown by an increase of the fishes' inclination. The degree of the change of central state is represented by the factor $U$ which is obtained by multiplying the altered sin $\beta$:sin $\alpha$ to its original value:

$$U = \frac{(\sin \beta) : (\sin \alpha)_{\text{before}}}{(\sin \beta) : (\sin \alpha)_{\text{after}}}$$

When $U$ exceeds 1.0, it signifies that the value for the optical stimulus has increased. Thus, $U$ gives a direct measure of the change of state, since the central turning tendencies which compensate for one another are directly proportional to sin $\alpha$ and sin $\beta$.

The black tetra was allowed to swim freely in a cylindrical cage (diameter 32 mm, length 75 mm) against a laminary water current of 1.0–1.5 cm/sec in the direction of the recording camera (Figure 1A and B). The front and back of the cage consisted of a grid mounted on a Plexiglas tube that was lying in a second Plexiglas tube (diameter 57 mm). The water flowed from the cage through the inner tube to the outlet. The closed system was placed in an experimental container at 25°C; the inner walls of the container were lined with black velvet, except for a circular opening at the front (diameter 65 mm) for recording, and a 25 × 18-cm horizontal split along the side where the light from two 100-W Osram bulbs impinged on the tank through a frosted glass. In each test, 0.7–0.8 ml of the solution under investigation was injected through a polyethylene tube (diameter 0.5 mm) with a 2-ml syringe. The solution reached the fish 1–2 sec after the injection. The tetra was allowed to adapt to the cage conditions for 10–15 min before testing.

During one session of 35–60 min, six to twelve experiments were performed at 5-min intervals. Subsequently the recordings were evaluated with a videorecorder (Grundig electronic color BK 401 or JVC/VCR model CR 6060 E). Single frames were selected from monitoring (Grundig electronic BG 61 T or Finnvideo VM 17 FI). Due to the laterally compressed body, the inclination position of the tetra could be determined easily and precisely within ±1°. Inclination was measured every 1.0 sec for the period 20–1 sec before the test substance was introduced; every 0.2 sec from 1 sec before until 5 sec after the introduction of the substance; and every 1.0 sec for the period of 5–30 sec after the substance under investigation had been presented. The mean of the angle of inclination was calculated from the data before the solution was introduced. It served as the reference point ($U$ = 1.0) for the reaction which followed as shown in the examples (Figure 2 and 3). The factor $U$ was determined for each change of the angle of inclination. The reactions of the fish were plotted on millimeter paper (examples in Figures 2 and 3); the maxima of the factors $U$ were shown in histograms (example in Fig. 4). A limit separating the effective substances

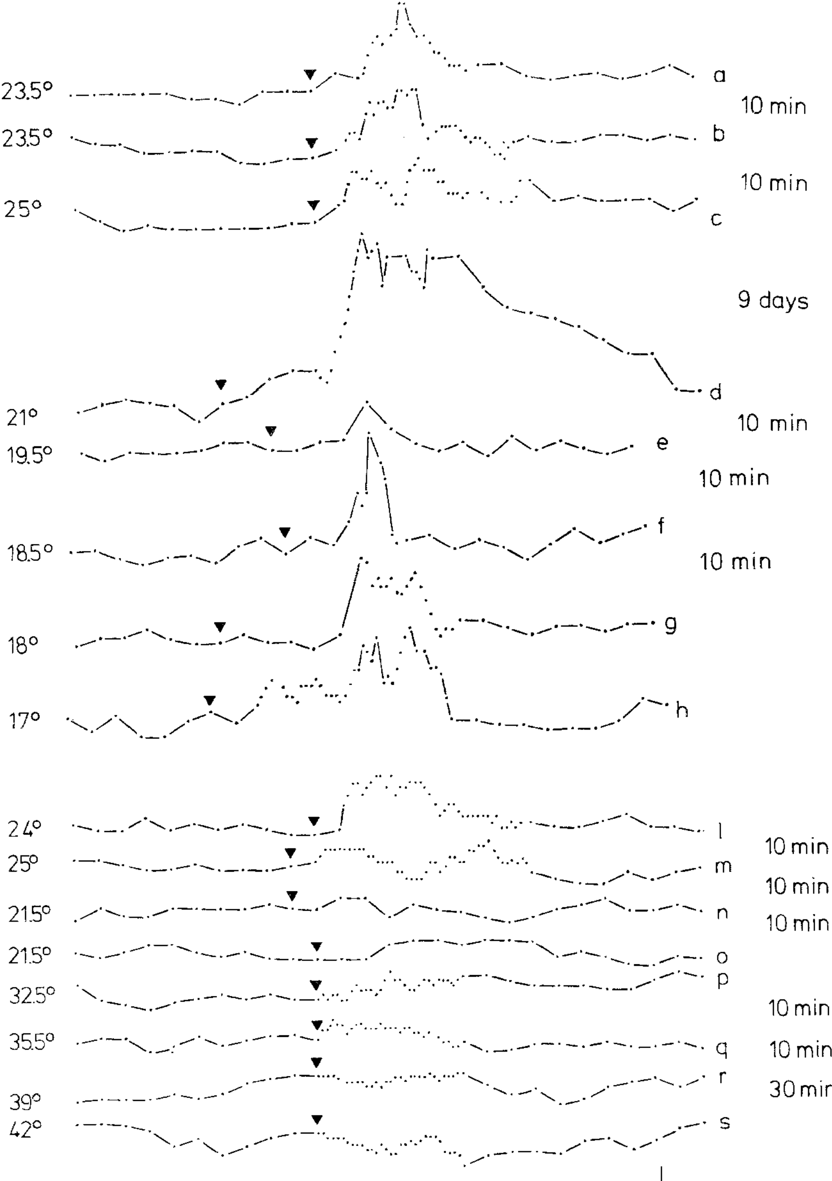


FIG. 2. "Change of central state" $U$ of *Gymnocorymbus ternetzi* elicited by skin extracts of conspecifics (a–h) and hypoxanthine-1($N$)-oxide (l–s). The median angle of inclination at the beginning of each experiment is shown at the left margin; for this angle of inclination $U = 1.0$. The interval between the experiments is shown at the right margin. The experiments are marked by small letters. The points (distance 1.0 resp. 0.2 sec) are calculated values for $U$ according to the angles of inclination measured; the lines make the curves easier to read. Abscissa: time (mark = 1.0 sec). Ordinate: $U$ (mark = 0.1 $U$). The arrow indicates the beginning of the addition of the substance, 1.5 sec after the start of the injection. a–g fish 11; h, fish 7.

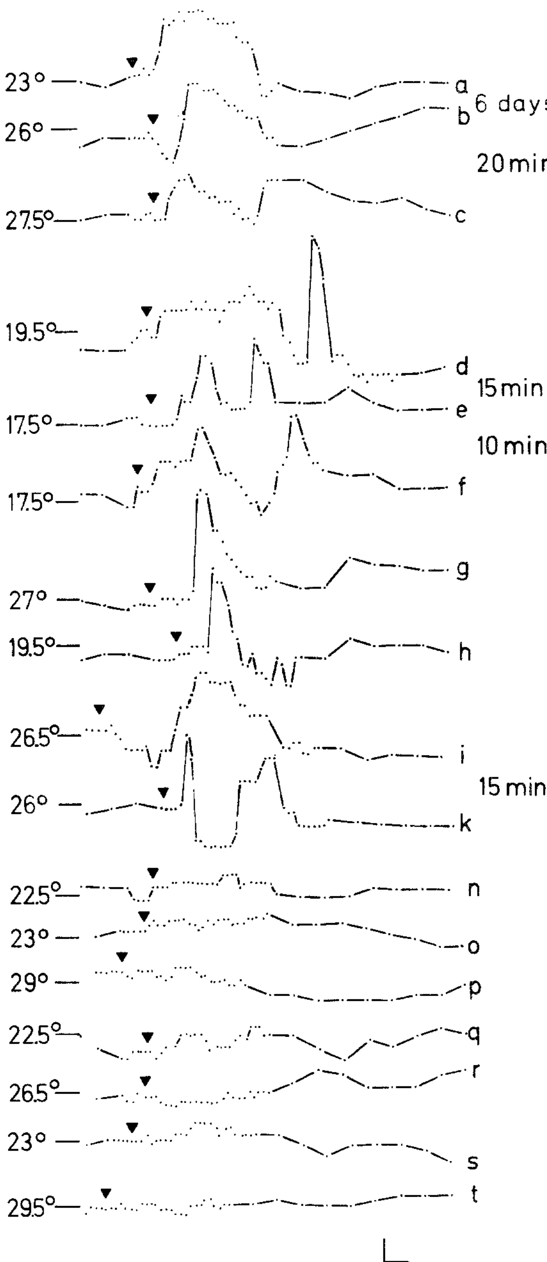


FIG. 3. Effects of hypoxanthine-3(*N*)-oxide (a–k) and hypoxanthine-1(*N*)-oxide (n–t) on the "change of central state" *U* of *Gymnocorymbus ternetzi*. a–c, fish 2; d–f, fish 1; g, fish 10; h, fish 4; i and k, fish 13. See Figure 2.

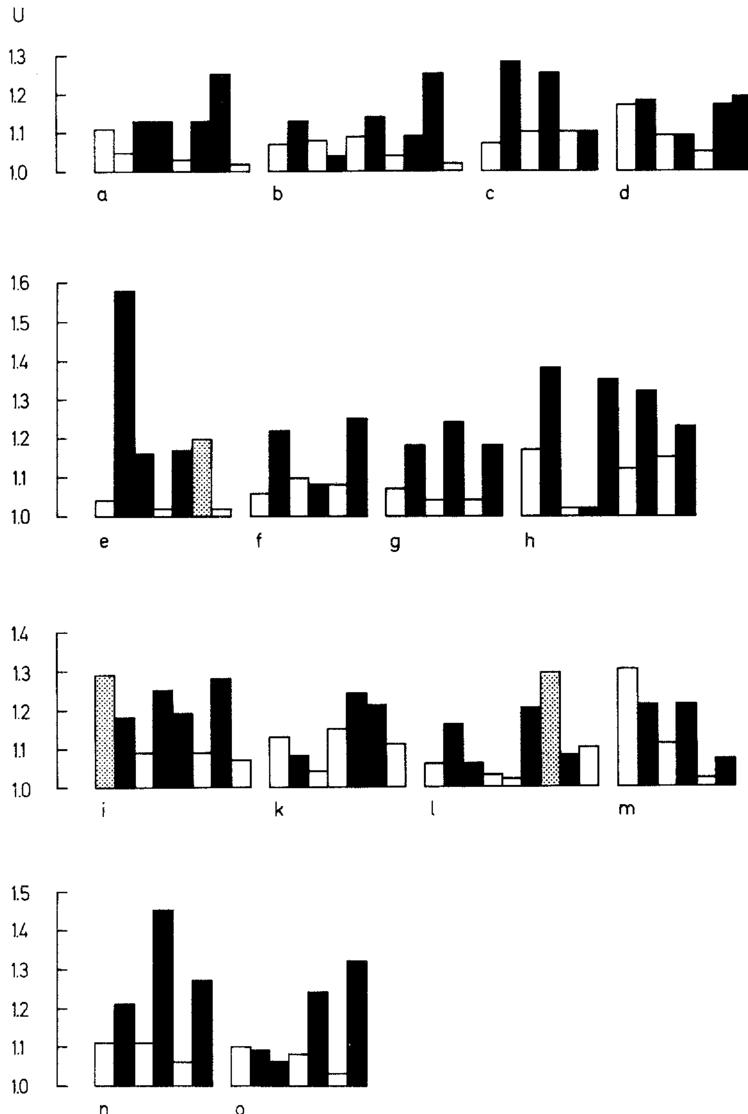


FIG. 4. Size of the "change of central state" $U$ (ordinate) of *Gymnocorymbus ternetzi* after stimulation with hypoxanthine-3($N$)-oxide ▬, hypoxanthine-1($N$)-oxide ▭, and stimulation with the visual dummy ▭. Every column represents one experiment. Experiments of one session follow one another every 4–5 min. The sessions are marked by small letters. Abscissa: successive experiments or, if separated from each other, successive sessions. (a) fish 2; (b) 6 days after (a); (c) 8 days after (b); (d) 6 days after (c); (e) fish 15; (f) 7 days after (e); (g) 6 days after (f); (h) fish 1; (i) fish 13; (k) 5 days after (i); (l) fish 16; (m) 7 days after (l); (n) fish 10; (o) fish 4.

TABLE 1. TWO-BY-TWO FREQUENCY TABLE SHOWING EXPERIMENTAL RESULTS WITH HYPOXANTHINE-1($N$)-OXIDE AND HYPOXANTHINE-3($N$)-OXIDE ON *Gymnocorymbus*

| | $U \geqq 1.15$ | $U < 1.15$ | Total |
|---|---|---|---|
| Hypoxanthine-1($N$)-oxide | 9 | 54 | 63 |
| Hypoxanthine-3($N$)-oxide | 52 | 18 | 70 |
| Total | 61 | 72 | 133 |

from the ineffective ones was chosen with $U = 1.15$ (Tables 1–4) and $U = 1.2$ (Tables 5–7), respectively.

Two groups of data resulted, one group above and one group below the limit. They were reported in two-by-two frequency tables (Tables 1–7), and their differences were examined for significance with Fisher's exact test. The values of $U$ elicited by different stimuli were compared by graphical methods (Figure 5). The effect of skin-extract from conspecifics (Characidae), and from the ide (*Leuciscus idus*; Cyprinidae), both containing alarm substance, was compared with hypoxanthine-3($N$)-oxide and hypoxanthine-1($N$)-oxide. Samples of 1 mg of each substance was dissolved in 10 ml 0.1 HCl and the solution

TABLE 2. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS OF EXPERIMENTS WITH HYPOXANTHINE AND HYPOXANTHINE-3($N$)-OXIDE

| | $U \geqq 1.15$ | $U < 1.15$ | Total |
|---|---|---|---|
| Hypoxanthine | 1 | 9 | 10 |
| Hypoxanthine-3($N$)-oxide | 11 | 0 | 11 |
| Total | 12 | 9 | 21 |

TABLE 3. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS IN EXPERIMENTS WITH SKIN EXTRACT FROM *Gymnocorymbus* AND HYPOXANTHINE-1($N$)-OXIDE

| | $U < 1.15$ | $U \geqq 1.15$ | Total |
|---|---|---|---|
| Skin extract from *Gymnocorymbus* | 0 | 7 | 7 |
| Hypoxanthine-1($N$)-oxide | 7 | 1 | 8 |
| Total | 7 | 8 | 15 |

TABLE 4. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS IN EXPERIMENTS WITH SKIN EXTRACT FROM *Gymnocorymbus* AND HYPOXANTHINE-3(*N*)-OXIDE

| | $U < 1.15$ | $U \geqq 1.15$ | Total |
|---|---|---|---|
| Skin extract from *Gymnocorymbus* | 0 | 7 | 7 |
| Hypoxanthine-3(*N*)-oxide | 0 | 9 | 9 |
| Total | 0 | 16 | 16 |

TABLE 5. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS IN EXPERIMENTS WITH 0.01 N HCl AND HYPOXANTHINE-3(*N*)-OXIDE

| | $U \geqq 1.2$ | $U < 1.2$ | Total |
|---|---|---|---|
| 0.01 N HCl | 0 | 14 | 14 |
| Hypoxanthine-3(*N*)-oxide | 18 | 7 | 25 |
| Total | 18 | 21 | 39 |

TABLE 6. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS IN EXPERIMENTS WITH HYPOXANTHINE-3(*N*)-OXIDE AND HYPOXANTHINE-3(*N*)-OXIDE + 10% $CaCl_2$

| | $U < 1.2$ | $U \geqq 1.2$ | Total |
|---|---|---|---|
| Hypoxanthine-3(*N*)-oxide | 2 | 12 | 14 |
| Hypoxanthine-3(*N*)-oxide + 10% $CaCl_2$ | 8 | 8 | 16 |
| Total | 10 | 20 | 30 |

TABLE 7. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS IN EXPERIMENTS WITH HYPOXANTHINE-3(*N*)-OXIDE AND HYPOXANTHINE-3(*N*)-OXIDE + 10% KCl

| | $U \geqq 1.2$ | $U < 1.2$ | Total |
|---|---|---|---|
| Hypoxanthine-3(*N*)-oxide | 5 | 5 | 10 |
| Hypoxanthine-3(*N*)-oxide + 10% KCl | 10 | 2 | 12 |
| Total | 15 | 7 | 22 |

TABLE 8. TWO-BY-TWO FREQUENCY TABLE SHOWING RESULTS OF BEHAVIOR EXPERIMENTS WITH HYPOXANTHINE-1(*N*)-OXIDE AND HYPOXANTHINE-3(*N*)-OXIDE ON SCHOOLS OF GIANT DANIO

| | Fright reaction | | |
|---|---|---|---|
| | + | – | Total |
| Hypoxanthine-1(*N*)-oxide | 0 | 37 | 37 |
| Hypoxanthine-3(*N*)-oxide | 17 | 14 | 31 |
| Total | 17 | 51 | 68 |

further diluted 1:10 with water. The solutions were marked by numbers only, and the experimenter did not know whether the substance under investigation was effective in previous experiments with schools or in other experimental sessions. All experiments were performed independently by at least three different persons.

In addition, in six specimens of *Gymnocorymbus* the otolith of one utriculus was removed surgically. The fish were narcotized with urethane and operated under a binocular microscope. The success of the operation was confirmed morphologically (method from Ahrens, 1950) after the physiological experiments had been completed. These fishes were allowed to swim in 60-liter aquaria under normal conditions, and skin extracts from conspecifics, hypoxanthine-3(*N*)-oxide, and hypoxanthine-1(*N*)-oxide were injected into their tanks after they had compensated for the loss of their otolith, and their behavior was observed or recorded.

Finally, hypoxanthine-3(*N*)-oxide and hypoxanthine-1(*N*)-oxide were tested with respect to their biological activity on schools of the giant danio, *Danio malabaricus* (Jerdon) (Table 8). These experiments were performed as described earlier (Pfeiffer 1978).

Both *N*-oxides for the early experiments were donated by Professor Dr. M. Viscontini (University of Zürich); those of the later experiments were contributed by Professor Dr. G. Häfelinger (University of Tübingen).

## RESULTS

Hypoxanthine-3(*N*)-oxide and hypoxanthine-1(*N*)-oxide were compared in 14 sessions and 99 separate experiments using seven individual fish (Figure 4). In contrast to hypoxanthine-1(*N*)-oxide, the experiments with hypoxanthine-3(*N*)-oxide almost always resulted in $U$ values that exceeded 1.15. This was confirmed in further experiments with five other individuals (Table 1). As shown

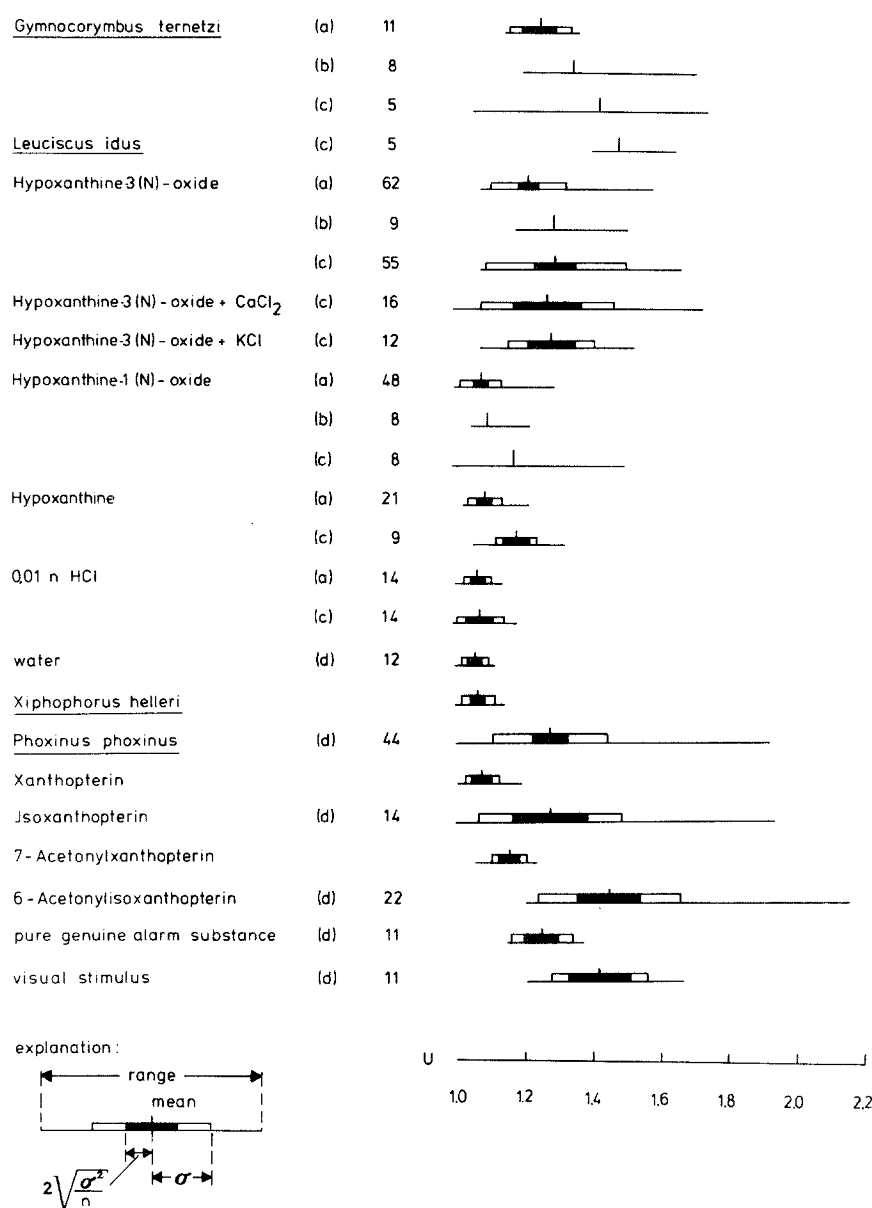


FIG. 5. Comparison of the size of the "change of central state" $U$ (abscissa) elicited by the different stimuli (ordinate), according to a method suggested by Hubbs and Hubbs (1953). n indicates the number of experiments; a, b, c show the data obtained independently by different members of our group. Represented are mean values, total range, standard deviation ($\sigma$ indicates dispersion) and 2 standard errors ($2\sqrt{\frac{\sigma^2}{n}}$, indicates degree of certainty). d data from Pfeiffer and Riegelbauer (1978) for comparison.

by the sharp ascent in the curve representing change of state (Figures 2 and 3), hypoxanthine-3(*N*)-oxide causes a clear change of state 2–4 sec after injection; the mean for $U$ was 1.30. Hypoxanthine-3(*N*)-oxide was, however, less effective than skin extract from the black tetra, *Gymnocorymbus ternetzi* ($\overline{U} = 1.43$) or from the ide, *Leuciscus idus* ($\overline{U} = 1.49$). Hypoxanthine-3(*N*)-oxide also proved highly effective when compared with hypoxanthine (Table 2), the 0.01 N HCl solution (Table 5), skin extract from *Gymnocorymbus* (Table 4), as well as from *Leuciscus* and an optical model (Figure 4).

Hypoxanthine-1(*N*)-oxide evoked, at best, a change of state below the borderline value of $U$ for spontaneous fluctuations (in this case, $U = 1.15$). In the graphic representation of change of state, there appear only insignificant fluctuations when hypoxanthine-1(*N*)-oxide is added; a definite increase in the value of $U$ is not apparent (Figure 3). A comparison between hypoxanthine-1(*N*)-oxide and hypoxanthine-3(*N*)-oxide in the two-by-two frequency test shows a significant difference (Table 1). Particularly clear is the comparison of hypoxanthine-1(*N*)-oxide with the skin extract of *Gymnocorymbus* (Table 3): only the latter is highly effective; for hypoxanthine-1(*N*)-oxide, the value of $U$ usually remains below 1.15, the mean for $U$ being 1.08. Thus, it is concluded that hypoxanthine-1(*N*)-oxide is inactive.

Skin extracts from the ide (*Leuciscus idus*, Cyprinidae) and from conspecifics (Characidae) were highly effective. While no significant difference between conspecific skin extract and hypoxanthine-3(*N*)-oxide could be established in the magnitude of the change-of-state factor in our initial experiments, further experiments indicated that the skin extract from *Gymnocorymbus* and *Leuciscus* were somewhat more effective than hypoxanthine-3(*N*)-oxide.

Mixtures of hypoxanthine-3(*N*)-oxide + 10% $CaCl_2$ or hypoxanthine-3(*N*)-oxide + 10% KCl proved no more effective than hypoxanthine-3(*N*)-oxide alone (Table 6 and 7). The mean change-of-state factors are in agreement and show that the mixtures are equally effective.

No evidence of adaptation was found. Neither stimulation with alarm substance nor hypoxanthine-3(*N*)-oxide resulted in adaptation; indeed, even repeated stimulation within only a couple of minutes was without effect.

The results for equilibrium behavior of *Gymnocorymbus* are completely in accord with the experimental results with schools of giant danio, *Danio malabaricus*, which showed that, in contrast to hypoxanthine-1(*N*)-oxide, hypoxanthine-3(*N*)-oxide elicited the fright reaction (Table 8).

The following examines the reactions of six specimens of *Gymnocorymbus ternetzi*, in which the otolith of one utriculus had been surgically removed. At first, a *Gymnocorymbus* with the left side surgically treated rotated towards the treated side around its longitudinal axis; this was interrupted by periods when the fish was found lying on its left side. After a few hours it swam almost normally with but a slight lateral inclination to the treated side. Lateral light exposure to the side of the operation induced a strong dorsal light response,

while exposure to the underside of the fish induced rotation around the longitudinal axis, towards the left side, with a frequency of 1–2 revolutions per second. Four to 12 days after the operation, the fish swam normally (also with light exposure to underside); it still reacted, however, to the capture net with rotation. The fish also responded to food (21, 26, 33, and 40 days after the operation) with rotation around the longitudinal axis, always towards the treated side. After 40 days the treated fish responded to conspecific skin extract with flight by means of rotation (recorded on videotape). This behavior could be aroused repeatedly; no evidence of adaptation was found. Morphological testing (method from Ahrens, 1950) confirmed the success of the operation (in this case, a missing left utriculus statolith). Corresponding results were achieved with five other unilaterally operated specimens of *Gymnocorymbus*; all individuals behaved uniformly. Whereas hypoxanthine-3(*N*)-oxide had the same effect as the alarm substance, hypoxanthine-1(*N*)-oxide was ineffective.

## DISCUSSION

The equilibrium behavior of *Gymnocorymbus ternetzi* proves suited for an investigation of the change of state in the central nervous system caused by the alarm substance. Only alarm substance and other substances (i.e., isoxanthopterin, 6-acetonylisoxanthopterin) which elicit the fright reaction in experiments on schools of giant danio, *Danio malabaricus*, produce a change of state. Increased optical excitation of the central nervous system manifests itself in a clear increase of the angle of inclination (inclination towards the light) for the unilaterally illuminated fish. In contrast, substances which did not elicit the fright reaction (i.e., xanthopterin, 7-acetonylxanthopterin) produced no substantial change in the angle of inclination of the fish and, as a consequence, no change of central state (Pfeiffer and Riegelbauer, 1978; Pfeiffer, 1978). This finding has now been confirmed for additional substances, namely, the highly effective hypoxanthine-3(*N*)-oxide and the ineffective hypoxanthine-1(*N*)-oxide (Figure 5). All the experimenters in our group obtained similar results (Table 1), which correspond to the findings on fish schools by the senior author (Table 8).

The factor *U* (von Holst, 1950a) can be used to measure a change of state in the central nervous system. The results achieved with various stimuli or directed towards various individuals can be compared by means of *U*, independently of the respective angle of inclination prior to stimulation. The use of a videorecorder permitted an exact drawing of the angle of inclination. The reactions of a fish in these experiments could be repeatedly observed on the monitor in consecutive frames separated by intervals of up to 1/50 of a second. In these short intervals, the angle of inclination could be exactly determined. Corresponding experiments by four different investigators enabled us to compare the results and to exclude all subjective impressions.

Habituation to the stimuli [genuine alarm substance or hypoxanthine-3(*N*)-oxide] was never apparent. The strength of the maximum change of state fluctuated independently of the number of times an individual was stimulated. The results correspond to the neurophysiological results of Schoen (1957): no adaptation occurs in recordings of single neuron discharge from the medulla oblongata and the cerebellum with incessant stimulation of the statolith. In behavior experiments with alarm substance, fish schools usually exhibit a quick habituation in the aquarium (von Frisch, 1941). This habituation could be the result of a higher center, which is influenced in its latter decisions by previous experience or learning. In a frightened school, only the motor flight response is visible; this response fails to appear after repeated stimulation. Presumably the alarm substance also triggers a change of state in the central nervous system of fish which no longer exhibit a fright reaction. This change of state was not visible since it was not expressed in any sort of motor response under the previous conditions applied.

Skin extract from *Phoxinus phoxinus* (Cyprinidae) and genuine pure alarm substance from this species were highly effective in *Gymnocorymbus ternetzi* (Pfeiffer and Riegelbauer, 1978). The same held true for conspecific skin extract (Characidae) and skin extract from *Leuciscus idus* (Cyprinidae) (Figure 5).

There is a fundamental difference between the present findings and the earlier data obtained by Pfeiffer and Riegelbauer (1978). Whereas neither isoxanthopterin nor 6-acetonylisoxanthopterin are identical with the genuine alarm substance (Pfeiffer, 1975, 1982), hypoxanthine-3(*N*)-oxide isolated by Argentini (1976) from the skin of the European minnow *Phoxinus phoxinus* is assumed to be a component of the alarm substance of the Ostariophysi or to be identical with this pheromone. The genuine alarm substance was isolated from this species in the laboratory of Professor Dr. M. Viscontini (University of Zürich), while all necessary behavior tests were performed by the senior author (University of Tübingen). The genuine product was highly effective in behavior experiments on schools of giant danio (*Danio malabaricus*) in 20-liter aquaria: at 0.05 mg, 8 of 10 experiments proved positive, at 0.005 mg, 15 of 18, and at 0.0005 mg, 10 of 19.

An investigation of the structure of the substance showed that it does not fluoresce and that it has a UV spectrum similar to that of purine. The substance remains stable in water for 24–26 h. The loss of UV absorption within 5 min after this time span points to an autocatalytic disintegration. In the mass spectrum, besides *m/e* = 81% of the basic peak, a smaller peak *m/e* 152 (4%) was found. The formula $C_5H_4N_4O$ could be ascribed to the peak *m/e* = 136. This formula corresponds to hypoxanthine. The genuine alarm substance and hypoxanthine showed identical spectra in $[^{13}C]$ NMR, but the substances were shown not to be identical in thin-layer chromatograms. In addition, hypoxanthine is known to exist in the skin of trout, which contains no alarm substance. Con-

sequently, hypoxanthine as such is not involved with the alarm substance. Furthermore, hypoxanthine proved ineffective in all 37 tests on schools of giant danio. Finally, in 30 tests on the black tetra, hypoxanthine elicited no change of state.

By means of [$^{13}$C] NMR, it has been verified that the alarm substance possesses the same C-structure as hypoxanthine. Therefore, one can assume that the peak *m/e* 152 in the mass spectrum represents the molecular peak of an *N*-oxide from hypoxanthine. Of the four possible structures 7(*N*)-oxide and 9(*N*)-oxide have not yet been found in nature; Argentini (1976), therefore, restricted his studies to 1(*N*)-oxide and 3(*N*)-oxide, especially since both natural substances show biological activity. Both substances were synthesized and identical $R_f$ values in thin-layer chromatographs with cellulose were found for hypoxanthine-3(*N*)-oxide and the genuine alarm substance from *Phoxinus*. The mass spectra were also identical. In particular, hypoxanthine-3(*N*)-oxide was effective in 17 and 31 experiments with schools of *Danio*, whereas hypoxanthine-1(*N*)-oxide was clearly negative in all 37 tests. Hypoxanthine-3(*N*)-oxide induced a change of state in *Gymnocorymbus*; hypoxanthine-1(*N*)-oxide was ineffective. These findings were independently collected by four persons working on different fish and with substances synthesized in two different laboratories.

Argentini (1976) had difficulties isolating alarm substance free of an inorganic residue from the skin of European minnow. The X-ray fluorescence method showed that this residue is composed of KCl and $CaCl_2$. A possible synergism of KCl and/or $CaCl_2$ with the organic component of the alarm substance cannot be excluded, especially since $CaCl_2$, according to Bodznick (1978), is a highly effective odorant to fish. The change of state caused by hypoxanthine-3(*N*)-oxide + $CaCl_2$ (16 tests), or hypoxanthine-3(*N*)-oxide + KCl (12 tests) lay in the range of the change of state which *N*-oxide alone elicited. Remaining to be investigated are the possible effects of ratios of $CaCl_2$ or KCl other than the 9 : 1 ratio previously used and also various combinations of the two salts.

In six specimens of *Gymnocorymbus ternetzi* which had compensated for removal of the statolith of one utriculus, conspecific skin extract or hypoxanthine-3(*N*)-oxide again triggered the same postoperative results, whereas hypoxanthine-1(*N*)-oxide was ineffective. Von Holst (1950a, b) sees this short-term decompensation as the charging up of the equilibrium center preceding situations accompanied by increased motor activity. According to this theory, the stimulus [here: alarm substance or hypoxanthine-3(*N*)-oxide] would prepare the equilibrium system for approaching demand; in other words, it would activate the system. If the equilibrium center is less excitable on one side, which is assumed to be the case in a fish in which the otolith of one utriculus has been removed, the activating excitation cannot evenly spread and leads to a strong predominance of the excitable side and thus to a turning tendency towards the surgically treated side. The perception of the alarm substance sets the fish into

an alarmed state, in which the equilibrium center is activated in anticipation of the imminent fright reaction. This leads to a turning tendency due to the difference in excitability of each side of the equilibrium center and, along with this, rotation along the longitudinal axis towards the treated side. The excitation revealed in this rotation prepares the fish for a flight response and the fright reaction. After only one day, the rotation response can again be aroused. Thus, perception of alarm substance not only activates the optic center (visible in the increase of the angle of inclination) but also activates the equilibrium center (visible in the rotation of a fish, in which the otolith of one utriculus has been removed). As the increase in the angle of inclination shows, the activation of the optic center is somewhat greater than that of the equilibrium center.

The results indicate that the increase of the equilibrium and especially the optical alertness is not a general response to odors but a very specific activation by substances that elicit the fright reaction, whether they are present in the fish's skin [like alarm substance and hypoxanthine-3(*N*)-oxide] or not (like isoxanthopterin and 6-acetonylisoxanthopterin). The process may be considered "CNS excitation by fear elicited by perception of the alarm substance" or short "fear excitation". This "fear excitation" seems to precede the flight response. The alarm substance prepares the static and especially the optic centers for the enhanced stress.

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# CONSTITUENTS OF MANDIBULAR AND DUFOUR'S GLANDS OF AN AUSTRALIAN *Polyrhachis* WEAVER ANT

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**Abstract**—Worker ants of *Polyrhachis (Cyrtomyrma) ?doddi* collectively discharge the secretions of their large mandibular glands when their nest is disturbed. The major glandular compounds of workers are 6-methylhept-5-en-2-one and phenylacetaldehyde oxime. Other components identified are mellein, 6-methylhept-5-en-2-one oxime, phenylacetonitrile, phenylacetaldehyde, benzaldehyde, and several alkanes and alkenes. The mandibular gland secretions of queens differ from those of workers only quantitatively. Large queens have considerably more of most components, the small queens have much less of volatile components. There is a pronounced sexual difference: the major components of the male's secretions are octanoic acid and mellein, with geranic acid, 8-heptadecene, 2-methylbutanoic acid, and 9-nonadecene present in lesser amounts. Workers of *P. ?doddi* also have unusually large Dufour's glands containing a large array of hydrocarbons, of which tridecane is the major component, but α-farnesene, pentadecane, and heptadecene are also present in large quantities.

**Key Words**—*Polyrhachis (Cyrtomyrma) ?doddi,* Hymenoptera, Formicidae, mandibular glands, Dufour's glands, defensive secretions, nitrile, oximes, mellein, 6-methylhept-5-en-2-one, benzaldehyde, phenylacetaldehyde.

## INTRODUCTION

The formicine species *Polyrhachis (Cyrtomyrma) ?doddi* is a weaver ant. The nest consists of sheets of larval silk woven between leaves and twigs and reinforced by soil and dead vegetable particles (Hölldobler and Wilson, 1983). The species selects a wide variety of bushes and trees as nesting sites.



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The workers forage individually, and colonies do not maintain exclusive territories. In fact, this *Polyrhachis* species is a docile ant that tends to avoid confrontations with other ant species, even when close to home. However, a slight vibrational disturbance of the nests, such as a tapping on the nest wall elicits an immediate outrush of a large number of ants that swarm over the nest and simultaneously discharge a strong-smelling substance. From the ants' behavior, we assume that this is a defensive response, possibly aimed at vertebrate predators, although other ant species, such as *Oecophylla smaragdina,* appear also to be repelled by *P.* ?*doddi* secretions. The defensive substance originates from the workers' large mandibular glands. In this paper, we report the results of our chemical analysis of its constituents. In addition, we have also studied the chemical components of the unusually large Dufour's gland of *P.* ?*doddi.*

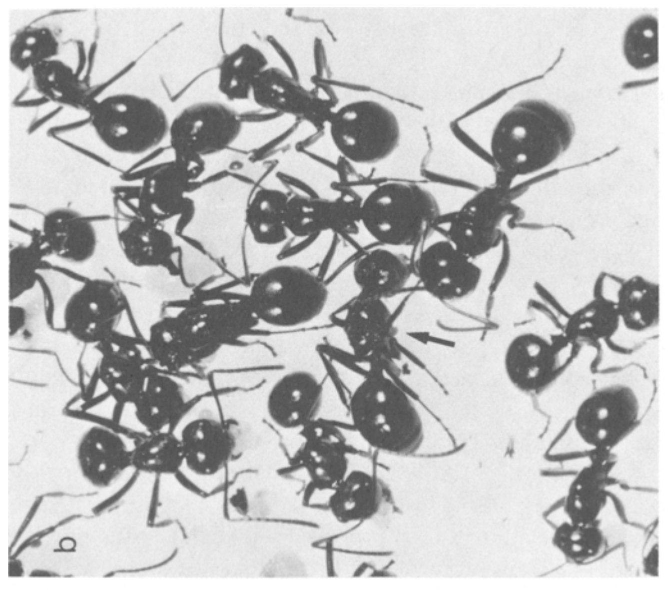
## METHODS AND MATERIALS

*Ants.* The species investigated was identified by R.W. Taylor as *Polyrhachis (Cyrtomyrma)* ?*doddi.* Voucher specimens have been deposited in the Australian National Insect Collection and in the Museum of Comparative Zoology of Harvard University. Colonies of the ants were collected near Port Douglas, North Queensland, and maintained in the laboratory for several months. The colonies were either left in their natural leaf nests or else housed in test tube nests containing natural nest material. Each colony had access to a foraging arena and was provided with honeywater and pieces of freshly killed cockroaches (*Nauphoeta cinerea*).

Each nest usually contained 100–400 workers, several (2–11) large queens (Figure 1A), and often a larger number (3–26) of small queens as well (Figure 1B). Approximately 86% of the large queens that we dissected proved to be inseminated, and all had fully developed, active ovaries (17–22 ovarioles); only 36% of the small queens were inseminated, and 58% had fully developed ovaries (14–17 ovarioles). In comparison, we never found inseminated workers; their ovaries consisted of only 2–4 ovarioles, the bases of which were often filled with a yellowish or orange liquid. Some colonies also contained males.

For histological investigations, live specimens were fixed in Carnoy's fluid, embedded in methyl methacrylate, and sectioned 8 μm thick with a D-profile knife on a Jung Tetrander I microtome (Rathmayer, 1962). The staining was Azan (Heidenhain). For additional morphological studies, the glands were dissected in Ringer solution and drawn to scale under a dissecting microscope.

*Extraction of Glandular Components.* In order to prevent the ants from discharging glandular secretions prematurely, they were chilled at 3–5°C for approximately 20 min. The glandular organs were then dissected out under water and placed in pentane for a few minutes. In order to obtain larger samples of the mandibular gland secretions, heads of the ants were cut from the body and crushed under pentane. After several minutes, the heads were removed to leave



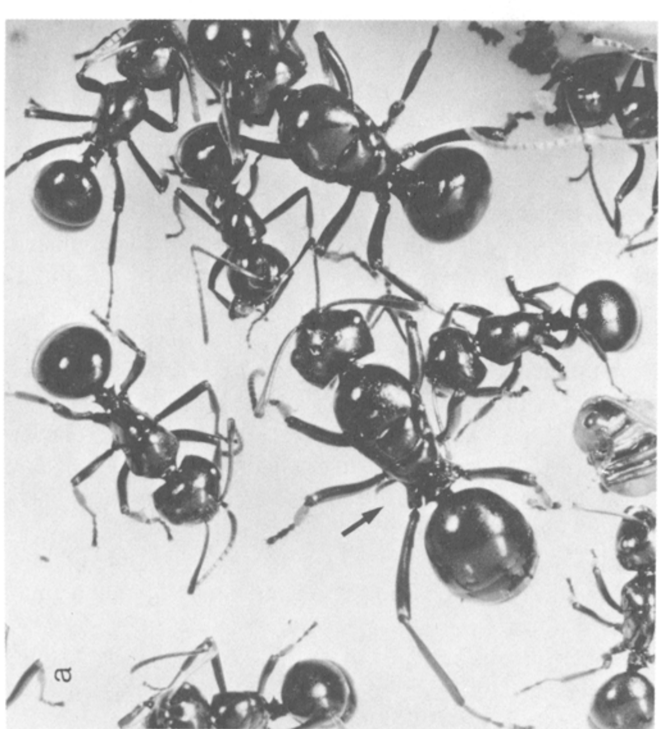




FIG. 1. *Polyrhachis (Cyrtomyrma) ?doddi* workers and queens. (a) Arrow points to large queens; (b) arrow points to small queen.

a solution of the secretion. All solutions of the secretions were stored in a freezer when not being investigated.

*Instrumentation.* Gas chromatography was conducted on a Varian aerograph model 1400 instrument equipped with glass columns and a flame ionization detector. Gas chromatography–mass spectrometry was conducted by directing some of the effluent through a jet separator to a VG Analytical model 70-70 mass spectrometer. Gas chromatographic columns employed were: column A, 5% OV-101 on 80–100 mesh Gas-chrom Q, 1.5m × 2mm; column B, 5% Carbowax 20 M–terephthalic acid terminated on 80–100 mesh Gas-chrom Q, 3 m × 2 mm; column C, same phase as column B but 1.5 m × 2 mm; and column D: 3% Superox 4 on 80–100 mesh Gas-chrom Z, 4 m × 2 mm.

Samples for ozonolysis were collected on a Varian aerograph model 1200 gas chromatograph fitted with a glass column 1.5 m × 2 mm. The phase was the same as for column A. The effluent from a 10:1 splitter was collected in glass capillaries 150 mm × 1.6 mm OD which were cooled with solid $CO_2$. Relative amounts of components were determined on a Varian aerograph model 2100 with glass columns 2 m × 3 mm packed with 5% OV-101 on Gas-chrom Q or 3% Superox 4 on Gas-chrom Z. The FID output was processed by a Varian model CDS111 integrator. No corrections were made for differences in detector response factors.

The relative amounts of selected components in extracts of workers and queens were determined from solutions prepared by crushing two heads of each category under pentane in the same manner as already described. The volume of the solution was adjusted to 100 $\mu$l. The relative amounts of 6-methylhept-5-en-2-one were obtained by injection of aliquots onto column A at 90°. The amounts of the others were determined by GC-MS with the mass spectrometer in selected ion monitoring (SIM) mode. Column B at 200° was utilized for the estimation of mellein (ion selected, $m/z$ 178) and the same column at 180° for the 6-methylhept-5-en-2-one oximes ($m/z$ 141), phenylacetonitrile ($m/z$ 117), and the phenylacetaldehyde oximes ($m/z$ 117).

Ozonolyses were carried out by passing ozonized oxygen through the capillary containing the sample still cooled by solid $CO_2$. After 1 min, the contents were washed from the tube with 5 $\mu$l of pentane which contained a little triphenylphosphine and the solution collected in a syringe. The sample was injected into the GC-MS system employing column A and examined. Products were identified from their retention times and from comparisons of mass spectra with the spectra of authentic aldehydes.

*Preparation of Compounds for Comparisons.* 6-Methylhept-5-en-2-ol was prepared from 6-methylhept-5-en-2-one by reaction with lithium aluminium hydride in dry ether.

Phenylacetaldehyde was prepared by oxidation of 2-phenylethanol with pyridinium chlorochromate in dichloromethane. The alcohol was obtained by reduction of ethyl phenylacetate with lithium aluminium hydride.

Oximes were prepared by shaking the carbonyl compound with an aqueous solution of hydroxylamine hydrochloride using NaOAc as buffer. The oximes were extracted into ether.

A mixture of geranic acid [(*E*)-3,7-dimethylocta-2,6-dienoic acid] and nerolic acid (*Z* isomer) was prepared from citral by oxidation with $Ag_2O$ in ethanol–water. Of the two components with molecular ions at *m/z* 168, ratio 1:4, the one in the larger amount was designated as geranic acid (Pickett et al., 1980).

*trans, trans*-α-Farnesene was obtained by washing each of three small Granny Smith apples with about 10 ml pentane. The volume of the combined washings was reduced to about 1 ml by boiling on a steam bath. This sample contained high boiling wax much of which precipitated from the cooled solution but α-farnsene, almost the only volatile component, remained in solution (Murray, 1969).

## RESULTS

*Morphology of Mandibular Glands.* Both workers and large queens have large mandibular glands whereas those of the small queens are considerably smaller. The proportionately largest mandibular glands are found in males, where the glandular sacs fill almost the entire side portion of the head capsule (Figures 2 and 3).

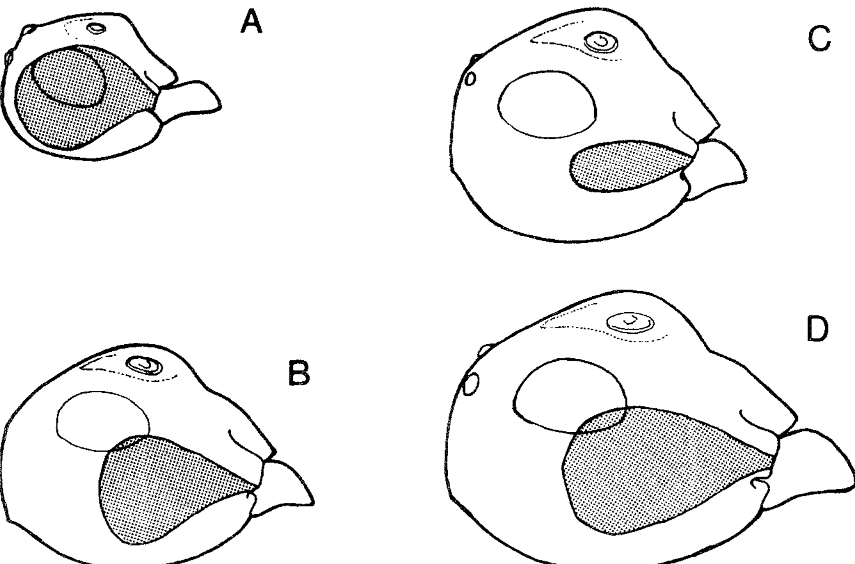



FIG. 2. Schematic illustration of the proportions of the mandibular glands (shaded area) of males (A), workers (B), small queens (C), and large queens (D).

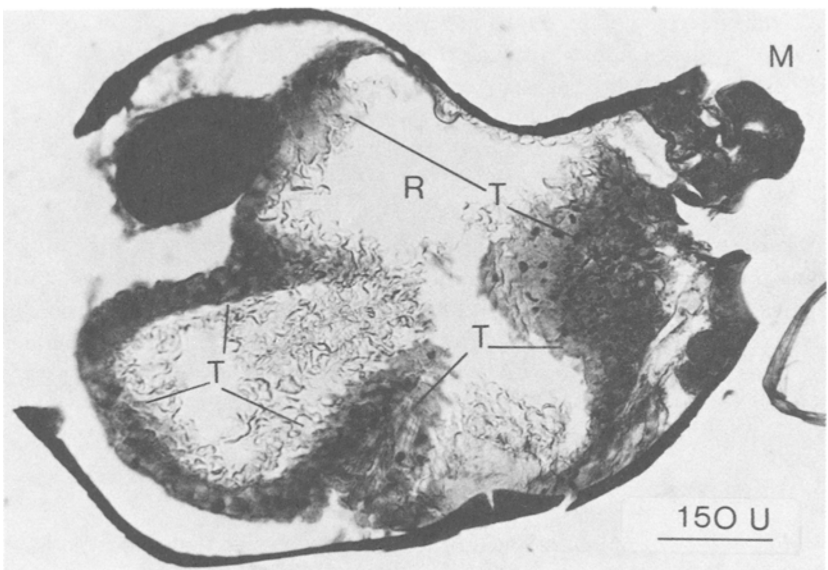



FIG. 3. Longitudinal section through the mandibular gland of a *P. ?doddi* male. M: mandible; R: glandular reservoir; T: glandular tissue.

*Secretions of Worker Mandibular Glands.* The extract obtained from the dissected mandibular glands was analyzed on column A, and the chromatogram obtained is shown in Figure 4. The pentane extract of crushed worker heads was a clear pale brown solution, and it gave a similar chromatogram.

The mass spectrum of the major component (1, 66.7% of volatiles) had a molecular ion at *m/z* 126 and a fragmentation pattern very similar to 6-methylhept-5-en-2-one, a common component of ants. The identity was confirmed by comparison with an authentic sample which had been extracted from *Iridomyrmex purpureus.* Component 1 matched the ketone in its mass spectrum and in its retention times on columns A and B.

Components 6 (11.8%) and 7 (14.4%) had similar mass spectra: component 6, *m/z* 135 (49%), 118 (42), 117 (90), 116 (11), 91 (100), 90 (43), 89 (15), 65 (18), and 51 (11); component 7, *m/z* 135 (40%), 118 (27), 117 (100), 116 (12), 91 (95), 90 (56), 89 (17), 65 (20), 63 (10), and 51 (11).

The odd-numbered molecular mass indicates the presence of one N; the loss of 17 and 18, the presence of a readily lost hydroxyl; and the group of peaks around 91, a benzylic residue. In addition metastable peaks for the transitions 135 → 117 (101.3), 118 → 91 (70.2), and 117 → 90 (68.2) could be assigned in both spectra. Preparation of the oximes of phenylacetaldehyde gave a product which matched components 6 and 7 in their mass spectra and their retention

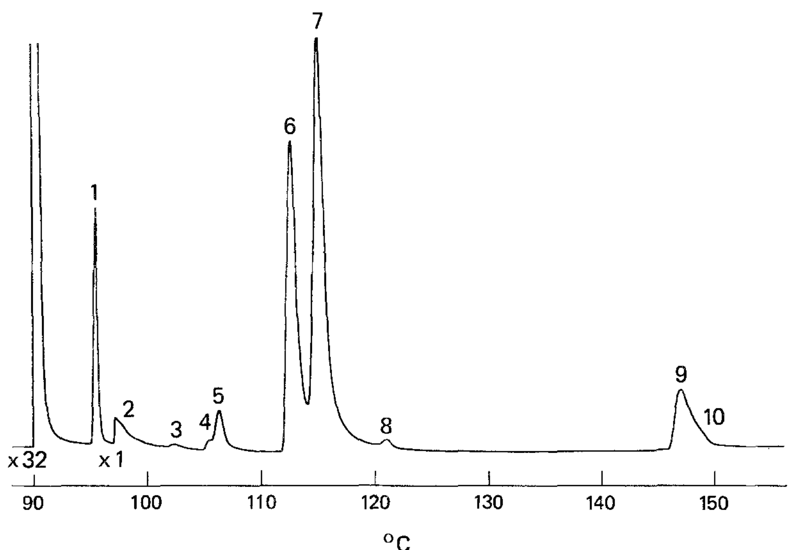



FIG. 4. Chromatogram of the pentane extract of mandibular glands of *P. ?doddi* workers. The phase was OV-101 and the temperature program from 90° at 4°/min. Compounds identified are 1, 6-methylhept-5-en-2-one; 2, phenylacetaldehyde; 3, phenylacetonitrile; 4 and 5, 6-methylhept-5-en-2-one oxime; 6 and 7, phenylacetaldehyde oxime; 8, tridecane; 9, mellein; 10, pentadecane.

times on column A. On column B the *E* and *Z* isomers were not resolved but matched in retention time the corresponding peak in the ant extracts.

Component 3 (0.3%) had a molecular ion of *m/z* 117; major fragments at 116 (30), 90 (80), and 89 (20); and was clearly related to components 6 and 7. It matched phenylacetonitrile in its mass spectrum and in retention times on columns A and B.

The mass spectrum of component 9 (3.5%) showed a molecular ion and the base peak at *m/z* 178 and major fragment ions at 160 (46), 149 (21), 135 (17), 134 (80), 132 (13), 106 (17), 105 (13), 104 (15), 78 (21), and 77 (16). This spectrum agreed with that reported for 3,4-dihydro-8-hydroxy-3-methyl-1H-2-benzopyran-1-one (mellein) which had been isolated from males of *Camponotus* spp. by Brand et al. (1973a). The identification was confirmed by comparison with an authentic sample which agreed in its mass spectrum and its retention time on column B at 180° (18.3 min).

Components 4 (0.4%) and 5 (1.4%) both showed molecular ions at *m/z* 141 and base peaks at *m/z* 41 but otherwise had significantly different fragmentation patterns: component 4, *m/z* 141 (1%), 126 (1), 124 (1), 82 (65), 73 (24), 69 (97), 67 (42), and 41 (100); and component 5, *m/z* 141 (28%), 126 (7), 124 (12), 109 (20), 98 (23), 83 (22), 82 (11), 73 (91), 69 (90), and 41 (100).

Once again the presence of a single N could be inferred. Component 5 showed a loss of methyl and the extended fragmentation suggested an aliphatic molecule with methyl branching. The loss of 17 (OH) suggested an oxime, and the presence in the secretion of large amounts of phenylacetaldehyde oximes and 6-methylhept-5-en-2-one with the possibility of transoximation prompted the preparation of the oximes of the ketone. The two products matched the ant components in their mass spectra and in the retention time on column B from which they emerged as one peak. The large differences in the mass spectra between the *E* isomer (component 5) and the *Z* isomer (component 4) arise from the different orientations of the hydroxyl of the oxime group to the methylene adjacent to the double bond (Whittle and Bellas, unpublished).

Component 8 (0.2%) agreed in its mass spectrum with tridecane, while component 10 (0.4%), better resolved on the 2100 instrument, agreed with pentadecane. The retention times also matched those of authentic samples under isothermal conditions on column A.

Component 2, barely discernible in Figure 4, with a molecular ion at *m/z* 120 (24%) and fragment ions at 92 (25), 91 (100), and 65 (19), matched phenylacetaldehyde in its retention times on columns A and B.

Injection of the extracts onto the Carbowax column showed the same major constituents except that on this column the *E* and *Z* oximes were not resolved. In addition components which matched benzaldehyde (*m/z* 106, 105, and 77) and 6-methylhept-5-en-2-ol (*m/z* 128, 110, 95, 81, 69, and 55) in their mass spectra and their retention times (column B, 100°; benzaldehyde 8.76 min, 6-methylhept-5-en-2-ol 5.89 min) were detected. Further hydrocarbons were identified from their mass spectra: molecular ions at *m/z* 240 (heptadecane), 238 (heptadecene), and 266 (nonadecene). All of these additional components occurred at concentrations lower than that of phenylacetaldehyde.

*Secretions of Mandibular Glands of Queens.* The mandibular gland secretions of the large queens resemble those of the workers but contain more of each component and for some components considerably more. The small queens had very much less of volatile material, but all of the major components were present in the extract. A comparison of relative amounts of several compounds in extracts of heads of the workers and the large and small queens is presented in Table 1.

*Secretions of Male Mandibular Glands.* Injection of the head extract onto column A showed the presence of two major components, about 40% and 30%, respectively, of total volatile material, and several minor ones, and the unsymmetrical shapes of three of the peaks showed these components to be very polar. The peaks had better shapes on column B at 160°.

The first major component (retention time on column B, 8.00 min) was polar. The mass spectrum showed a molecular ion at *m/z* 144 (3%) and significant fragment ions at 127 (1), 115 (12), 101 (26), 86 (15), 85 (25), 84 (23), 73 (73), and 60 (100). The mass spectrum agreed with that of octanoic acid, and

TABLE 1. AMOUNTS OF SELECTED COMPONENTS IN LARGE AND SMALL QUEENS AND WORKERS RELATIVE TO AMOUNT OF 6-METHYLHEPT-5-EN-2-ONE PRESENT IN WORKERS

| | Workers | Large queens | Small queens |
|---|---|---|---|
| 6-Methylhept-5-en-2-one | 1000 | 7900 | 200 |
| Phenylacetonitrile | 4 | 5 | 0.5 |
| 6-Methylhept-5-en-2-one oximes | 26 | 44 | trace |
| Phenylacetaldehyde oximes | 390 | 490 | 27 |
| Mellein | 53 | 127 | 6 |

this acid had the same retention time as the ant component on column B. The second major component, with retention time 35.7 min, matched mellein. Retention times (18.30 min) on column B at 180° were the same for the corresponding component of workers and of large queens.

A polar component which emerged at 1.80 min had a molecular ion *m/z* 102 (1%) and fragment ions at 87 (28), 74 (100), and 57 (59), and the spectrum agreed well with that of 2-methylbutanoic acid. The retention time of the acid was 1.82 min under the same conditions, and the two substances were considered to be the same.

One component (retention time 2.28 min, $M^{+\cdot}$ *m/z* 238) matched heptadecene, while another (retention time 4.80 min, $M^{+\cdot}$ *m/z* 266) matched nonadecene. Ozonolysis of the first hydrocarbon gave octanal and nonanal, and of the second, nonanal and decanal. Thus the double bonds are nine carbons from one end in both, and the compounds are 8-heptadecene and 9-nonadecene, respectively.

The remaining sizable component at a retention time of 23.00 min was polar, and the mass spectrum showed a molecular ion at *m/z* 168 (3%) and fragment ions at 153 (1), 151 (1), 125 (5), 123 (11), 100 (17), 82 (6), 69 (100), and 41 (47). The spectrum was a good fit for either geranic or nerolic acids (Pickett et al., 1980). Comparison on column C at 160° with a mixture of nerolic and geranic acids prepared from citral showed that the ant component matched geranic acid (retention times: geranic acid, 12.71 min; nerolic acid, 10.75 min; ant component, 12.75 min).

*Secretions of Dufour's Glands of Workers.* As in most other formicine ants, the Dufour's gland of our *Polyrhachis* species is bilobed. However, the gland is considerably larger than in most formicine species, stretching almost to $\frac{1}{3}$ the length of the gaster (Figure 5). Only the slave-making species *Formica pergandei* and *F. subintegra,* which use large amounts of Dufour's gland secretions as propaganda pheromones during slave raids, have proportionately larger Dufour's glands (Regnier and Wilson, 1971).

The volatile components of the extract were analyzed by GC-MS on col-

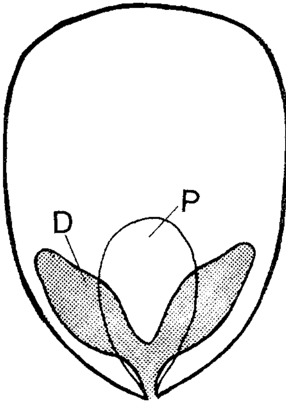



FIG. 5. Schematic illustration of the proportions of the Dufour's gland (D) and poison gland (P) and gaster of *P. ?doddi* workers.

umns A and D. All the compounds identified were hydrocarbons, and their structures were assigned from their mass spectra and from Kovats index measurements on column A (Bergström and Löfqvist, 1973). The compounds identified and their relative proportions are listed in Table 2. Several other components were present in quantities too small to allow positive identification. The major components are tridecane, α-farnesene, pentadecane, and heptadecene. The location of double bonds was not investigated.

The α-farnesene of the ant was compared with a sample of *trans, trans*-α-farnesene which was isolated from Granny Smith apples. The mass spectra were almost identical and the retention indices were: column A, (130°) 1496, 1495; and column D (100°), 1728, 1728 for the ant and apple farnesenes, respectively. On column A, the peak due to pentadecane underlaid the peak of the α-farnesene, but its presence appeared to have little effect on the index measured.

## DISCUSSION

Workers of the weaver ant *Polyrhachis (Cyrtomyrma) ?doddi* have large mandibular glands, the secretions of which are readily discharged by defending workers when their nest is mechanically disturbed. Our chemical analyses reveal that the secretions consist of a rich blend of components, some of which are well known from ants, while others have not been detected in the Formicidae.

To the former group belong 6-methylhept-5-en-2-one and its corresponding alcohol, which have been found in the pygidial glands (anal glands) of several dolichoderine species (see review by Blum and Hermann, 1978) and in the mandibular glands of formicine ants, including *Lasius fuliginosus* (Bernardi et al.,

TABLE 2. VOLATILE COMPOUNDS OF DUFOUR'S GLAND OF WORKERS OF *Polyrhachis ?doddi*[a]

| Compound | Retention index | Relative amount |
|---|---|---|
| Undecane | 1100 | 23 |
| Dodecane | 1200 | 22 |
| Tridecene[b] | | 5 |
| Tridecane | 1300 | 1000 |
| 5-Methyltridecane | 1454 | 9 |
| 3-Methyltridecane | 1471 | 14 |
| Tetradecane | 1400 | 7 |
| Pentadecene | 1478 | 60 |
| Pentadecane[c] | | 220 |
| α-Farnesene | 1496 | 670 |
| Hexadecene | 1578 | 8 |
| Heptadecene | 1678 | 170 |
| Heptadecane | 1700 | 28 |
| Octadecene | 1774 | 4 |
| Nonadecene | 1872 | 98 |
| Nonadecane | | 4 |

[a] Some of the components were not detected during measurement of the retention indices. Amounts are expressed relative to tridecane (=1000) and are derived from measurements on chromatograms off OV-101 and Superox columns.
[b] Two components with a molecular ion at $m/z$ 182 were observed on the Superox column.
[c] Pentadecane was not resolved from α-farnesene on OV-101.

1967), *L. carniolicus* (Bergström and Löfqvist, 1970), *Calomyrmex* sp. (Brown and Moore, 1979) and *Polyrhachis simplex* (Hefetz and Lloyd, 1982). Benzaldehyde has been previously detected in the secretions of the pygidial gland of the dolichoderine *Azteca* sp. (Wheeler and Blum, cited in Blum and Hermann, 1978) and in the mandibular gland of the myrmicine *Veromessor pergandei* (Blum et al., 1969). Mellein is known from mandibular gland secretions from males of several *Camponotus* species (Brand et al., 1973a,b; further data in Blum, 1981). In *P. ?doddi* we found it in females as well as in males. The presence of alkanes and alkenes in the mandibular gland secretions was surprising to us; but Brophy et al. (1982), studying the head extracts of another Australian *Polyrhachis* species, also found high-molecular-weight hydrocarbons. In fact, the hydrocarbons seem to constitute the major contents of the secretions in that species.

Phenylacetaldehyde, phenylacetonitrile, 6-methylhept-5-en-2-one oxime, and phenylacetaldehyde oxime have not, to our knowledge, been reported from ants before. The characteristic odor of the mandibular gland secretions is due to the presence of phenylacetonitrile. Even nest material retained this odor after removal of the ants. The pentane extract of nest material showed a component

with an ion at 117 at a retention time close to that for the nitrile. However, the peak was very small, being visible only on the SIM trace from the mass spectrometer.

Phenylacetonitrile and phenylacetaldehyde oxime have been identified from the millipede *Harpaphe haydeniana,* but they are present only in trace amounts. Duffey and Towers (1978) have shown that both compounds constitute metabolic intermediates in the production of mandelonitrile, which functions as a cyanogenic precursor in this millipede and in others (Eisner et al., 1963a,b; Towers et al., 1972).

The mandibular gland secretions of the queens appear to differ from those of the workers only quantitatively. In particular, they contain more of each component and for some components considerably more. The small queens, although approximating the size of workers, had very much less of volatile material. Taylor (personal communication) suggested the possibility that the small queens belong to another species, conceivably a social parasite. In fact, one of us (B.H.) observed and photographed in the field a small queen as she was attempting to enter a *P. ?doddi* nest and was attacked and held by resident workers.

We found a pronounced sexual difference in the constituents of the mandibular gland secretions. The main components of the male's secretions are octanoic acid, mellein, geranic acid, 8-heptadecene, and 2-methylbutanoic acid. Sexual differences in the mandibular gland secretions in ants are not unusual. Law et al. (1965) reported qualitative differences for *Lasius* and *Acanthomyops;* differences were also found in several *Camponotus* species (Brand et al., 1973a, b, see review in Blum, 1981), in a *Calomyrmex* species (Brown and Moore, 1979), and Pasteels et al. (1980) reported quantitative differences between males, queens, and workers of *Tetramorium caespitum.* The very large mandibular glands of *P. ?doddi* males and the distinct chemical profile of their secretions suggest that they play a significant role in sexual activities, possibly in producing mating aggregation pheromones and pheromones involved in sexual competition (see Hölldobler and Bartz, 1984).

Workers of *P. ?doddi* also have unusually large Dufour's glands containing a large array of hydrocarbons. Tridecane is present in the greatest amount, but $\alpha$-farnesene, pentadecane, and heptadecene are also represented in large quantities. These results are comparable with those obtained with another Australian *Polyrhachis* species in which Brophy et al. (1982), using whole gaster extracts, detected tridecane as the major compound, pentadecane as a second, and undecane as a third major compound. Somewhat different results were obtained with *Polyrhachis simplex,* in which Hefetz and Lloyd (1982) found 90% of the Dufour's gland secretions to be tridecane and only minor amounts to consist of undecane, dodecane, pentadecane, and heptadecane.

In many formicine species, the Dufour's gland secretions function as alarm

pheromones. Neither crushed Dufour's glands nor mandibular glands elicited alarm behavior in *P. ?doddi*. We do not yet know what the function of the large Dufour's glands in this species is. *Polyrhachis ?doddi* workers rarely attack by biting with their mandibles. When an individual ant is disturbed, it usually freezes in a typical position with the head held downward and the gaster bent forward beneath the thorax. But when the nest is disturbed, the ants discharge massive quantities of the mandibular gland secretions so that the air reeks of the characteristic smell. This strongly suggests that the mandibular gland secretions of *P. ?doddi* are employed in chemical defense.

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# AMMONIA UTILIZATION BY THE BRUCHID BEETLE, *Caryedes brasiliensis* [BRUCHIDAE]

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**Abstract**—The seed predator, *Caryedes brasiliensis* [Bruchidae] generates appreciable ammonia in its dietary use and detoxification of L-canavanine and its catabolic product, L-canaline. L-Canavanine is a toxic allelochemical of *Dioclea megacarpa* seeds, the food of the developing larvae. Bruchid beetle larvae rely upon glutamic acid dehydrogenase and glutamine synthetase to use ammonia for glutamic acid synthesis from 2-oxoglutaric acid and conversion of the former to glutamine. These reactions provide the larvae with a means for metabolically eliminating ammonia. Proline serves as a carbon skeleton source for glutamic acid formation.

**Key Words**—Ammonia utilization, L-canavanine catabolism, *Caryedes brasiliensis,* Coleoptera, Bruchidae.

## INTRODUCTION

The bruchid beetle, *Caryedes brasiliensis* [Bruchidae] inhabits the Neotropical deciduous forests of Costa Rica where it is the sole insect predator of the seed of the leguminous vine, *Dioclea megacarpa* (Janzen, 1971). This legume is distinctive in its storage of massive amounts of the highly insecticidal nonprotein amino acid, L-canavanine (Rosenthal, 1977a); it constitutes 55% of every nitrogen atom stored in the seed and accounts for about 95% of nitrogen allocation to free amino acid production (Rosenthal, 1977b).

*Caryedes brasiliensis* has achieved a number of biochemical adaptations that not only enable this seed consumer to utilize canavanine as a major dietary source of nitrogen (Rosenthal et al., 1982) but also to detoxify L-canaline, the highly deleterious breakdown product of canavanine utilization (Rosenthal,



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1978). *Caryedes brasiliensis* contains arginase (Rosenthal and Janzen, 1983), and it is also a prodigious urease producer (Rosenthal et al., 1976). These enzymes mediate canavanine conversion to canaline and urea and the breakdown of the latter to carbon dioxide and ammonia:

$$\underset{L\text{-}canavanine}{H_2N-(C-NH_2)=NH-O-CH_2-CH_2-CH(NH_2)COOH} \rightarrow$$

$$\underset{L\text{-}canaline}{H_2N-O-CH_2-CH_2-CH(NH_2)COOH}$$

$$+ \underset{urea}{H_2N-(C=O)-NH_2} \rightarrow CO_2 + 2NH_3$$

L-Canaline, a potent toxin to insects (Rosenthal, 1982a), is detoxified by this seed predator by converting L-canaline to L-homoserine and ammonia (Rosenthal et al., 1978). Since canaline is the predominant reaction product of canavanine catabolism, deamination of canaline increases ammonia production from canavanine by almost one half.

How does this seed consumer deal with the appreciable ammonia generated by its consumption of L-canavanine? This is an important question since ammonia can be toxic to insects, particularly terrestrial forms (Chefurka, 1965), and relatively little is known of its metabolism. *Caryedes brasiliensis* eliminates its ammonia by excretion directly into its fecal matter as the ammonium salt of organic acids. Analysis of the contribution of ammonia, urea, and uric acid to the total nitrogen of the frass disclosed that uric acid accounts for only 11% of the fecal nitrogen (Rosenthal and Janzen, 1981). Does this seed predator also eliminate ammonia by metabolic reactions, and what are these reactions? Automated amino acid analyses of the hemolymph (the circulatory fluid) of bruchid beetle larvae revealed the presence of appreciable proline and glutamine. These hemolymph analyses suggested that this seed-eating beetle might have an active glutamine synthetase (EC 6.3.1.2), an enzyme that fosters the reaciton of L-glutamic acid and ammonia to form L-glutamine (Tate and Meister, 1969). Additional removal of ammonia could be achieved by the reaction of 2-oxoglutaric acid (a tricarboxylic acid cycle intermediate) with ammonia to form L-glutamic acid. This reaction is catalyzed by glutamic acid dehydrogenase (EC 1.4.1.2). Working in concert, these reactions would provide a means of using ammonia to produce glutamic acid and then further using ammonia via glutamine formation.

## METHODS AND MATERIALS

*Insects.* The bruchid beetle larvae used in this study were obtained from infected *D. megacarpa* seeds collected in 1983 and 1984 in Santa Rosa National Park, Guanacaste Province, Costa Rica. The remaining insects were obtained

either from colonies maintained at the University of Kentucky or were the gift of Dr. George Kennedy, University of North Carolina, or Dr. Paul Feeny, Cornell University. The hymenopterous insects were collected from feral colonies in Kentucky. All insects were stored at −60°C prior to use.

*Enzyme Assays.* Unless otherwise indicated, terminal stadium larvae (2–2.5 g) were ground with a mortar and pestle utilizing about 8 ml of 100 mM *N*-Tris (hydroxymethyl)methylglycine (tricine) buffer (pH 7.3) and acid-treated sea sand. After the resulting slurry was centrifuged at 18,000*g* for 15 min, floating debris was removed from the supernatant solutions by filtering over cheesecloth.

The insect homogenates were assayed for glutamine synthetase activity at 37°C by the method of Rowe et al. (1970) except for omission of 2-mercaptoethanol. Zero-time samples served as the control. Glutamic acid dehydrogenase activity was monitored by evaluating the oxidation of NADH at 340 nm at 22°C. The assay mixture included 25 mM sodium 2-oxoglutarate (pH 7.3), 50 mM tricine (pH 7.3), 0.36 mM NADH, and ammonium chloride (pH 7.3) in a final volume of 1 ml. Ammonium-free samples served as the control. The apparent $K_m$ values of Table 1 are for ammonia in the glutamic acid dehydrogenase reaction. Activity is expressed in as nanomoles product formed per minute at the indicated temperature per milligram souble insect protein. Soluble protein values were determined by the method of Lowry et al. (1951).

## RESULTS AND DISCUSSION

Analyses of the catalytic activity of these two enzymes from 16 insects in addition to *C. brasiliensis* have been conducted (Table 1). While all of the tested insects contain detectable glutamine synthetase activity, *C. brasiliensis* larvae possess twice the enzyme activity of any of the tested insects and over 40 times more than the American cockroach, *Periplaneta americana;* the saltmarsh caterpillar, *Estigmene acraea;* and the Colorado potato beetle, *Leptinotarsa decemlineata.*

Comparable examinations of the glutamic acid dehydrogenase activity indicate the disproportionately high activity of this enzyme in the larvae of *C. brasiliensis.* With the exception of *Drosophila melanogaster,* the glutamic acid dehydrogenase activity runs from 3.5 times more than in *Callosobruchus maculatus,* a closely related beetle, to 34 times more than in *Polistes* spp., a wasp. This enzyme was not detected in four of the examined invertebrates. The glutamic acid dehydrogenase assay is sufficiently sensitive to detect less than 1 nmol $NAD^+$ formation. The enzyme activity values of Table 1 indicate product formation per unit of time. It is not known presently if this is a reflection of the intrinsic amount of the enzyme or the rapidity with which it converts substrate to product, i.e., its turnover number.

TABLE 1. GLUTAMINE SYNTHETASE AND GLUTAMIC ACID DEHYDROGENASE OF VARIOUS INSECTS[a]

| Organism | Enzyme activity | | |
|---|---|---|---|
| | Glutamine synthetase | Glutamic acid dehydrogenase | Apparent $K_m$ |
| | (nmol/min/mg) | | (mM) |
| Diptera | | | |
| *Drosophila melanogaster* | 25.4 | 108 | 600 |
| *Musca domestica* | 11.5 | 71 | 400 |
| Orthoptera | | | |
| *Periplaneta americana* | 0.8 | 13 | 190 |
| Hymenoptera | | | |
| *Vespula* spp. | 8.1 | 35 | 230 |
| *Polistes* spp. | 13.0 | 8 | 105 |
| Lepidoptera | | | |
| *Manduca sexta* | 10.9 | 49 | 430 |
| *Papilio polyxenes asterius* | 4.3 | 28 | 290 |
| *Ephestia kühniella* | 9.3 | ND | – |
| *Heliothis zea* | 10.6 | ND | – |
| *Heliothis virescens* | 14.9 | ND | – |
| *Hyphantria cunea* | 21.6 | 16 | 210 |
| *Galleria mellonella* | 17.2 | ND | – |
| *Estigmene acraea* | 1.0 | 21 | 190 |
| Coleoptera | | | |
| *Tribolium castaneum* | 18.6 | 33 | 245 |
| *Callosobruchus maculatus* | 21.2 | 78 | 240 |
| *Leptinotarsa decemlineata* (adult) | 1.2 | 48 | 160 |
| *Caryedes brasiliensis* (adult) | 12.7 | 62 | 345 |
| *Caryedes brasiliensis* | 49.5 | 272 | 70 |

[a]ND, denotes organisms lacking detectable enzymatic activity.

Another important parameter of enzyme function is indicated by the apparent Michaelis-Menten constant ($K_m$) of its substrate. This constant is inversely proportional to the enzyme–substrate affinity. Insectan glutamic acid dehydrogenase is characterized by a high apparent $K_m$ for ammonia (Table 1). This enzyme clearly exhibits limited affinity for ammonia. In several instances, the $K_m$ value was in excess of 300 mM and in *Drosophila melanogaster,* it reaches 600 mM. Except for *Polistes* spp., all of the tested insects other than *C. brasiliensis* larvae possess a $K_m$ for ammonia on the order of 200 mM. In *Polistes* spp., although the $K_m$ is low, enzyme activity is among the smallest of values obtained in this study. Only in the case of the larvae of the bruchid beetle are both the enzyme activity elevated and substrate affinity high. These two

factors suggest that this seed predator is competent at reacting ammonia with 2-oxoglutaric acid to form glutamic acid.

It is particularly interesting to compare these data for adult *C. brasiliensis* with those for the larvae. Adult *C. brasiliensis* are pollen and nectar feeders that do not consume canavanine-containing plant material. Adult bruchid beetles are very similar to the other tested insects both with regard to their glutamine synthetase and glutamic acid dehydrogenase activities (Table 1).

The combined action of these enzymes provides a means for moving the carbon skeleton from the reaction intermediates of the tricarboxylic acid cycle to glutamic acid and then to glutamine. However, in the event of appreciable ammonia production, these reactions can deplete the energy-producing intermediates of the tricarboxylic acid cycle. This potentially deleterious situation can be circumvented by glutamic acid formation from proline. *Caryedes brasiliensis* stores appreciable proline in its hemolymph (Rosenthal, 1983); it is a predominate component of the free amino and imino acids. Proline in the hemolymph represents a significant carbon skeleton pool for glutamic acid production without depleting tricarboxylic acid cycle intermediates. Certain insects can convert proline to glutamic acid as part of their energy-yielding reactions. Proline can be oxidized to $\Delta^1$-pyrroline-5-carboxylic acid, spontaneously converted to glutamic acid 4-semialdehyde, and then transferred to glutamic acid. The last reaction supports insectan bioenergetics by generating reduced NAD from $NAD^+$.

L-[U-$^{14}$C]Proline (0.5 $\mu$Ci/larva) was injected parenterally into 48 terminal instar larvae (3.88 g fresh weight); the larvae were sacrificed after 18 hr. The treated larvae were defatted by grinding in freshly distilled acetone and their free amino and imino acids isolated and purified by ion-exchange chromotography (Rosenthal, 1982b). The carbon-14 of the free amino and imino acids of the hemolymph was determined by automated amino acid analyses and postcolumn evaluation of the radioactivity of the various ninhydrin-positive compounds by liquid scintillation spectroscopy (Bray, 1960).

These analyses revealed that after 18 hr, 75% of the injected radioactive proline remained as proline. This limited metabolism of $^{14}$C-labeled proline may result from dilution of the labeled proline by the appreciable cold proline of the hemolymph. Of the radioactive proline that was converted to amino acids, nearly 70% was found as glutamic acid and glutamine. Thus, proline does function to provide some of the carbon skeleton required for the metabolism of ammonia.

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## *Book Review*

**Mammalian Semiochemistry. The Investigation of Chemical Signals Between Mammals.** Eric S. Albone. With a contribution from Stephen G. Shirley. Chichester: A Wiley–Interscience Publication, John Wiley & Sons Ltd., 1984. $57.00, 360 pp.

Within the last 30 years or so, the interest in behavior of animals has intensified tremendously, and, as a consequence of this, the need emerged to study how they communicate by means of odor signals. Subsequently a large number of articles, books, and proceedings of symposia devoted to the topic of chemical communication in mammals have been published by biologists, physiologists and ethologists, while chemists were reluctant to express their viewpoints. Now at last the silence has been broken, and we are in possession of a valuable volume that summarizes up-to-date achievements and exposes the role of the chemist in this manifold field of biology. This is a very useful book, especially for biologists engaged in the study of olfactory communication in mammals, since it helps them to better understand the immense problems that their collaborating chemists have to face.

Over 600 references covering various aspects of olfaction will be most useful not only to beginners but also to specialists who are already well established in the field of mammalian semiochemistry. The text of the book is organized into nine chapters that acquaint the reader with a wide range of topics covering the field of olfactory communication. There is also an appendix in which description of basic chemical methods available for studies of volatiles are given. This will also be of special help to nonchemists.

In the first chapter the author emphasizes the interdisciplinary nature of the studies of semiochemical signals and explains the terms most frequently used.

In the second chapter, after stressing the chemical complexity of odors, the question of volatility is discussed, and descriptions of methods available for sampling odorous materials are given.

The third chapter, based predominantly on information available concerning humans, emphasizes the uniqueness of the skin. Its histology is described, including different types of glands and hormonal control of their secretions. The occurrence of steroids in the skin is the subject of a separate section. The chemistry of skin surface lipids has been studied in considerable detail in man. In-



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formation gathered is compared with that available for seba in various species of mammals. In the appendix to this chapter, abbreviated notations of lipid components are given.

Chapter 4, the longest in the book, is devoted to odoriferous glands of mammals. Selected examples involving a large number of species representing five orders (Artiodactyla, Lagomorpha, Proboscidea, Rodentia, and Carnivora) illustrate a wide range of morphological features, behavioral involvements, and chemical compositions. A separate section deals with glands in primates and man.

The surface of the skin, and particularly glands secreting into pouches and skinfolds provide an ideal environment for various forms of microorganisms, which may contribute towards formation of semiochemical signals from vaginal, oral, axillary, skin, and anal gland secretions as well as from feces. The role of microorganisms in shaping of odor profiles is discussed in chapter 5.

The communicative function of urine odor is particularly well known because of extensive studies of its manifold effect on reproduction and sexual development of the domestic mouse and other rodents. In the sixth chapter, the author, after giving examples of behavioral function of urine, deals with fundamentals of its chemistry in humans and animals.

It has been frequently suggested that secretions derived from different parts of the reproductive tract—notably vaginal discharge—may possess semiochemical properties. Chapter 7 gives useful basic information relevant to this topic.

The odor of saliva seems to play a behavioral role in many species of mammals but has been adequately studied only in the domestic pig. Chapter 8 discusses this topic.

For a complete comprehension of communication by means of olfaction, it is important to understand how an odor signal is perceived by an animal. The last chapter (9) entitled "Mammalian Chemoreception," written by Stephen G. Shirley, provides information concerning physiology of the olfactory system and speculation on how it works. A view of utmost importance is expressed in the last sentence of this chapter. Following the emphasis that a mammal, after receiving the odor signal, has to process information at a higher level in the nervous system, the author concludes: ". . . it is by discarding the pheromonal type of receptor that detailed communication becomes possible."

I personally feel that although the multidisciplinary character of the problem is clearly evident throughout the text, the future task of histologists could be stressed more strongly. There is ample evidence that the behavioral status of an individual is very convincingly reflected not only in the size but also in histological appearance of its odoriferous glands. Histochemists have an important role to play in elucidation of formation of odor signals.

Albone's message to fellow chemists is that the field of mammalian

semiochemistry is wide open but may be even more difficult than insect semiochemistry has turned out to be.

This volume is recommended not only to chemists and biologists who contemplate studying the olfactory behavior or mammals, but also to those who are generally interested in the behavior of animals and complexities of nature. The easy-flowing style makes it enjoyable reading.

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*Journal of Chemical Ecology, Vol. 11, No. 4, 1985*

## *Book Review*

**Pheromones and Reproduction in Mammals.** John G. Vandenbergh (ed.). New York: Academic Press, 1983. $39.50, 298 pp. (ISBN: 0-12-710780-0).

The appearance of this authoritative volume by ten of the leading researchers in the field of pheromones and mammalian reproduction is a timely reminder of how far our understanding of this topic has advanced in just over two decades. The book is essentially a series of reviews of what is known about pheromones in the signaling and priming context of mammalian reproduction.

Robert Johnston discusses scent marking in mammals as a phenomenon related to the estrous cycle. He discusses an odor-quality model for reproductive communication quite unlike the one-pheromone–one-receptor model thought to hold for insects, one that is additive while being nonlinear. Michael Leon, in a thoughtful chapter, examines the role of pheromones in mother–young interactions and shows how well-suited pheromones are to effect communication. Natural selection appears to have modified the mechanisms which regulate mothers' nonlactating physiology to the task of regulating their interactions with their offspring in a very parsimonious manner. The final chapter on signaling pheromones, by Eric Keverne, examines the part played by odors in the reproductive biology of primates. Traditionally regarded as microsmatic on the anatomical evidence of olfactory lobes, Keverne shows that olfactory cues are important in primate reproduction, but how the information which male monkeys clearly receive about their consorts is used is still an open question.

John Vandenbergh discusses the role of the olfactory environment on the timing of the onset of sexual reproduction in female rodents and shows that puberty is controlled by a variety of acceleratory and inhibitory signals. He draws attention to a most significant point: until further work is conducted upon natural populations of known size and density, the real significance of the painstaking laboratory work cannot be assessed. Martha McClintock takes Vandenbergh's chapter further by examining the pheromonal regulation of the estrous cycle. Cycles can be shortened or lengthened by changes wrought in the development of follicles, the timing of surges of gonadotropins, or the persistence of the corpus luteum. The unanswered question still is, how do pheromones actually regulate these activities? Only time will tell.

Anna Marchlewska-Koj examines the block to pregnancy effected by strange male odor and again questions whether the phenomenon is widespread



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in nature and whether it is able to affect population dynamics. Frank Bronson and Bruce Macmillan show that the secretory patterns of LH and prolactin are altered by the olfactory environment, as are the steroids which are dependent upon them, but once again field evidence is sparse.

The penultimate chapter, by Michael Meredith, reviews the sensory physiology of pheromone communication and pays particular attention to the functioning of the vomeronasal organ. Although this structure, of widespread occurrence among mammals, is undoubtedly involved, Meredith wisely counsels against the assumption that all chemosensory information is processed by this and the olfactory system. The final picture may be much more complex.

The final chapter is an examination of the applied end of olfactory biology—the involvement of pheromones in the reproductive biology of domestic mammals. The success of the artificial use of pig pheromones in normal husbandry is well known; M.K. Izard suggests that similar practices may have a role to play in the management of other species in the future, for the benefit of all mankind.

Every chapter of this book provokes a great many unanswered questions, and that is as it should be. In this rapidly advancing field, new material is being added to the stock of knowledge daily, and reviews like this are a vital link between researchers, for a question raised in one field may help provide an answer in another. For my money, I'd have liked to see an attempt at the construction of some basic concepts in olfactory biology in a form sufficiently coherent and compact as to be immediately available to readers other than diehard afficionados. As it is, I do not think this book will find its way onto the shelves of many population ecologists, and that is a pity because the time is now at hand when ecologists and physiologists must explore together to see if theory is upheld in practice.

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# *Announcements*

## INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY

The 2nd Annual Meeting of The International Society of Chemical Ecology will be held on June 8-12, 1985 on the campus of The University of Wisconsin, Madison, Wisconsin. Program highlights will include symposia on "Chemical Aspects of Symbiosis: Multitrophic Level Considerations," and "Behavioral Adaptations to Chemical Cues," and in the general subject areas of "chemical ecology of aquatic environments" and "insect allomones." The official call for submitted papers and posters, along with forms for pre-registration and lodging and meal reservations, will go out to members in early March 1985. Limited funds will be available for defraying expenses of students participating in the meetings. All inquiries should be directed to Dale M. Norris, 642 Russell Labs, University of Wisconsin, Madison, WI 53706. Telephone number (608) 262-3626.

Future meetings are planned tentatively for Berkeley, California in 1986 and outside the U.S. in 1987.

## PLANT/HERBIVORE INTERACTIONS

The Shrub Research Consortium is sponsoring the fourth wildland shrub symposium August 12-14, 1985 at Snowbird Resort, near Salt Lake City, Utah. The symposium, "Plant/Herbivore Interactions," will feature invited and contributed papers on aspects of plant-animal interactions with an emphasis on but not limited to vertebrate herbivores and shrub ecosystems. Contributed presentations will be 20 minutes. The proceedings will be published. If you would like to present a paper, send a title and abstract by May 15, 1985, to:

Dr. F. D. Provenza
Department of Range Science
College of Natural Resources
UMC 52
Utah State University
Logan, Utah 84322

For further information about the symposium and facilities, please contact:

Theresa A. Bigbie
Conferences and Workshops
Brigham Young University
297 CONF
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# INHIBITION OF BARNACLE SETTLEMENT AND BEHAVIOR BY NATURAL PRODUCTS FROM WHIP CORALS, *Leptogorgia virgulata* (LAMARCK, 1815)[1]

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**Abstract**—Laboratory-reared barnacle larvae were used to study natural products from whip corals. Biological assays used barnacle responses in behavioral assays and in larval settlement assays. Whip corals contained substances that were active in both assays. Substances inhibiting swimming and reversible attachment of barnacle larvae were found in an aqueous extract of whip corals. Low-molecular-weight substances inhibiting barnacle settlement were found in a methylene chloride extract of material soluble in aqueous methanol. Antisettlement activity was associated with substances with specific mobilities in several chromatography systems. Partially purified material was effective in preventing settlement at concentrations of less than 0.2 $\mu$/ml. Settlement inhibition involves adsorption of the natural product to surfaces.

**Key Words**—Barnacle settlement, natural products, gorgonians, settlement inhibition, *Balanus amphitrite, Leptogorgia virgulata,* fouling, antifouling.

## INTRODUCTION

Whip and fan corals, gorgonians, are common members of some benthic communities. Their conspicuous growth form and apparent lack of mechanical defenses has resulted in speculation that they use chemical defenses (Burkholder and Burkholder, 1958; Burkholder, 1973; Bernstein et al., 1974; Cimino and De Stefano, 1978; Perkins and Ciereszko, 1973; McEnroe and Fennecal, 1978; Hadfield and Ciereszko, 1978). Although there are specific assemblages of organisms associated with gorgonians (Patton, 1972), common fouling organisms such as algae, balanoid barnacles, oysters, and bryozoans are not included. This



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lack of fouling may result from antifouling compounds produced by the gorgonial (Burkholder and Burkholder, 1958; Ciereszko, 1962; Cimino and De Stefano, 1978; Hadfield and Ciereszko, 1978).

Standing et al. (1984) tested a common subtropical gorgonian, *Leptogorgia virgulata* (Lamarck), for substances that inhibited settlement of the barnacle, *Balanus amphitrite* Darwin. The dialyzable fraction of an aqueous homogenate of *L. virgulata* soft tissue contained active material. We report more detailed studies of barnacle settlement inhibitors from *L. virgulata*. We show that the low-molecular-weight fraction of gorgonian homogenate contains at least two types of inhibitory substances. One type affects larval behavior. The other type, including at least two substances, inhibits settlement. We have concentrated on the latter substances and present procedures for their purification. We also present experimental data supporting the hypothesis that inhibition is via a surface-modification mechanism.

## METHODS AND MATERIALS

### *Culture of Larval Barnacles*

*Balanus amphitrite* cyprids were cultured from stage 1 nauplii as described by Rittschof et al. (1984). The day of metamorphosis to cyprid was called day 0. Cyprids were stored in the dark at 4°C until the day of use.

### *Biological Assays*

*Behavior Assay.* Behavior assays were performed as described by Rittschoff et al. (1984). Briefly, a 120-cm × 3.7-mm ID Pyrex tube was used as an assay chamber. A low (15–17%) level of larval attaching behavior was promoted by coating chambers with six 10-sec rinses of a 250 ng protein/ml solution of settlement factor derived from *Balanus amphitrite* homogenate (Lahrman et al., 1982; Rittschof et al., 1984). A high level of attaching behavior (50–70%) was promoted by coating the chamber with a 2.5$\mu$g protein/ml solution of settlement factor. In uncoated tubes 5–7% of cyprids exhibited sticking behavior. Cyprids were placed in the upstream 10 cm of the tube, and flow generating a velocity gradient (Crisp, 1955) of 39 $sec^{-1}$ was initiated. Flow was stopped after 10 min, the tube drained at a velocity gradient of 606 $sec^{-1}$. Cyprids remaining in the tube were scored as stickers, those flushed from the tube upon draining were counted as sliders. Cyprids that passed through the tube in the first 10 min were scored as swimmers.

*Settlement Assay.* In settlement assays, 25–50 3-day-old cyprids/replicate were incubated for 20 hr at 25°C and a 15 : 9 light–dark light regime (11 : 9 light–dark in tests) in covered polystyrene Petri dishes (Falcon 1006) (Branscomb and Rittschof, 1984; Rittschof et al., 1984). Test compounds were added to filtered seawater (< 100,000 daltons). The residue from a water sample taken through

the fractionation procedure was run as a control. Experimental solutions were made by dissolving vacuum-dried test samples in control seawater. From two to seven concentrations of test solution, each with three to six replicates, were tested by comparing frequency of permanent attachment (shown as percentage settlement) in experimental solutions to attachment in the controls.

*Preparation of Assay Samples.* Assays testing effects of solvents showed that cyprid settlement was inhibited by small amounts (1$\mu$l methanol/ml for example) of organic solvents. Therefore, samples for bioassay were dried under vacuum to remove solvents. After drying, samples were diluted in seawater with concentrations expressed as milligrams original animal homogenized per milliliter seawater. Experiments testing the inhibitory effects of solvents dried in the same fashion showed drying eliminated settlement inhibition caused by solvents.

*Reagents and Supplies.* Solvents were reagent grade or purer. Methanol was HPLC grade from Fisher, methylene chloride and chloroform were reagent grade from Fisher Scientific. Methylene chloride was redistilled prior to use. Analytical thin-layer plates (microscope slide size) were: (1) silica with indicator (Whatman MK6F; (2) reverse-phase silica with indicator (Whatman MKC18F). Preparative silica TLC plates with indicator were Whatman PK6F. Reverse phase Sep-Pak® cartridges were from Waters Corporation.

*Collection and Processing of Soft Coral*

*Leptogorgia virgulata* colonies were collected from subtidal sites in the vicinity of the Duke University Marine Laboratory. All color variants were represented in the proportions in which they occurred. Living colonies were cut into short lengths as they were collected. Samples were processed immediately through the low-molecular-weight (LMW) fractionation procedure (below) or divided into 100-g portions, packaged in freezer bags, frozen at −50°C, and stored at −20°C until processed.

*LMW Fractionation.* Fresh or frozen tissue samples (300–700 g) were mixed 1 : 1 w/v with water purified by reverse osmosis (R-O) and homogenized for 2 min in a seven-speed Waring blender at a setting of 7. Axial skeleton settled out. Homogenized soft tissues were decanted into centrifuge bottles and centrifuged at 6000*g* for 10 min. The supernate was transferred immediately to lyophilization flasks, shell-frozen, and lyophilized. Solids were resuspended in 50-75 ml R-O water and dialyzed against two 500- to 750-ml changes of water. The dialysate was lyophilized, and the solids were mixed with 100% methanol and centrifuged at 10,000*g* for 10 min. the supernate, a clear yellow solution, was termed the low-molecular-weight (LMW) fraction.

*Sep-Pak Fractions.* Sep-Pak $C_{18}$ cartridges were conditioned by washing at 1 ml/min with at least 20 ml of 100% methanol. Cartridges were equilibrated to application conditions prior to application of material to be fractionated. Ma-

terial was applied to the cartridges at a flow of 1 ml/min. Capacity experiments showed that substantial leakage of inhibitory substances could be expected if the equivalent of more than 40 g of original homogenate was applied to a cartridge. After application, the cartridge was washed with 10 ml of application solvent and then eluted with 10-ml volumes of increasing concentrations of methanol in R-O water. Eluates were concentrated to near dryness by rotary evaporation.

*Solvent extraction.* Preparation of inhibitor was simplified once inhibitory substances were detected by their characteristic migration in two TLC systems. After homogenization, centrifugation, and lyophilization, material was resuspended in 100% methanol at a concentration of 2–6 mg of original/ml and the resultant slurry centrifuged at 10,000*g* for 10 min. The clear yellow supernate was diluted to 25–40% methanol with water, filtered to remove a cloudy precipitate, and extracted with 3 × 1/4 vol of methylene chloride. The solvent extract was evaporated to dryness under vacuum, redissolved in a small amount of methanol or methanol–methylene chloride (100 g of original/ml). This material was used in preparative scale purifications.

*Preparative Scale Procedures.* Solvent extract was applied to preparative TLC plates in a 0.5 × 20 cm band at the origin. Plates were developed with 95:5 chloroform–methanol, and zones made visible by quenching of shortwave UV fluorescence. Zones were scraped off plates and eluted with small volumes of methanol.

### *Statistical Analysis*

Analyses were dependent upon the design of the experiment and the question being asked. Contingency analysis was used to compare frequencies in behavior essays (Sokal and Rohlf, 1981). Initially, analysis of variance was used to determine effects of test substances in settlement assays. Later, contingency analysis on frequencies of barnacles settling and not settling was used to compare control and test substances. Experiments were repeated at least two times to confirm statistical conclusions. Concentrations required for 50% inhibition (with 95% confidence limits) were calculated by the probit technique of Finney with a BASIC program for microprocessors written by Lieberman (1983).

### *Experiments*

The goals of the experiments were to: (1) confirm the existence of low-molecular-weight natural products from soft corals that function as settlement inhibitors; (2) characterize inhibition using settlement and behavior assays; (3) investigate the mechanism of action of settlement inhibitor; and (4) chemically isolate and characterize settlement inhibitor.

Specifically, tests determined: (1) if cyprid behavior and settlement were inhibited by low-molecular-weight fractions from soft coral; (2) if behavior and

settlement inhibition could be separated; (3) means for concentrating and purifying inhibitory substances; and (4) a method for preparative-level isolation of settlement inhibitors.

## RESULTS

### *Activities in LMW fraction of* L. virgulata

*Behavior Inhibition.* The low molecular weight fraction of *L. virgulata* was tested for behavior effects (Table 1). Assay tubes were rinsed in a 250 ng protein/ml solution of settlement factor to promote a low level of sticking behavior. Test substances were dissolved in seawater and were passed through the tubes at a flow rate of 7.5 ml/min. Cyprids in the control assay (without addition to seawater) responded to flow and surface reproducibly and in high percentage (72%) by swimming. Smaller percentages of cyprids (13%) slid down the tube or actively attached (15%) by their antennae (stickers). When not swimming, cyprids retracted their thoracic appendages. Exposed to a concentration of 20 mg original/ml of LMW, most cyprids (13%) slid down the tube. As compared to control, there was a significantly reduced frequency of swimmers and stickers and a significantly increased frequency of sliders (Table 1). Cyprids appeared to be immobilized, and did not completely retract their thoracic appendages when not swimming. Tests with a concentration of 5 mg original/ml gave results intermediate to control and 20 mg original/ml and significantly different from both (overall $G = 261.92$, $P < 0.005$). Cyprids in 5 mg/ml appeared normal upon microscopic investigation, but responded significantly less by swimming and sticking and significantly more by sliding than controls (Table 1). In some preparations, inhibitory activity (increased sliding) was observed at concentrations of 0.5 mg original/ml.

*Settlement Inhibition.* Settlement assays were used to test for settlement inhibition actively in the LMW fraction. LMW significantly inhibited settlement at concentrations of 20 and 10 mg original/ml and was ineffective at 5 mg/ml (Table 2). Although some variability in inhibitory activity between *L. virgulata*

TABLE 1. MODIFICATION OF LARVAL BEHAVIOR BY LMW

| | Total (%) | | | |
|---|---|---|---|---|
| Behavior | Control 1, 335[a] | Control 2, 290[a] | LMW 20 mg/ml, 354[a] | LMW 5 mg/ml, 482[a] |
| Swimmers | 72 | 72 | 12 | 60 |
| Sliders | 13 | 13 | 88 | 34 |
| Stickers | 15 | 15 | 0 | 5 |

[a]Number of animals.

TABLE 2. INHIBITION OF BARNACLE SETTLEMENT BY LMW[a]

| Treatment | Set | Not set | Set (%) | $G^b$ | $P$ |
|---|---|---|---|---|---|
| Control | 63 | 92 | 41 | | |
| 20 mg/ml | 26 | 118 | 18 | 17.56 | <0.001 |
| 10 mg/ml | 39 | 109 | 26 | 6.34 | <0.010 |
| 5 mg/ml | 75 | 115 | 39 | 0.01 | NS |

[a]$EC_{50}$ = 16.608 mg/ml (95% confidence interval 26.3–12.5).
[b]Statistical comparisons are to control.

LMW preparations was observed, inhibitory activity was always observed at a concentration between 2 and 10 mg of original/ml.

*Sep-Pak Fractions*

*Behavior Inhibition.* LMW fraction was applied to a $C_{18}$ Sep-Pak in water, washed with water, and eluted with 80% methanol. The effluent and 80% methanol eluate were tested for behavior and settlement inhibition activity. Tests of the Sep-Pak effluent and the 80% methanol eluate of adsorbed substances showed that behavior inhibitor was not adsorbed on the cartridge (Table 3). As reported in the case of the LMW, the aqueous effluent caused significant increase in the numbers of sliders and significant decreases in the numbers of swimmers and stickers. The 80% methanol eluate had no effect on behavior at the same relative concentration. Behavioral responses to the 80% eluate were not significantly different from the effluent (overall $G = 122.07$, $P < 0.005$).

*Settlement Inhibition.* Sep-Pak effluent and 80% methanol eluate of LMW were tested in settlement assays to determine location of settlement inhibition activity (Table 4). There was no detectable inhibition of settlement by the effluent at 32 mg original/ml. Effluent at 8 mg/ml significantly increased settle-

TABLE 3. LOCATION OF BEHAVIOR INHIBITION IN SEP-PAK FRACTIONS

| | Total (%) | | |
|---|---|---|---|
| Behavior | Control 1,875[a] | Sep-Pak effluent, 677[a] | Sep-Pak eluate, 539[a] |
| Swimmers | 28 | 9 | 24 |
| Sliders | 20 | 53 | 24 |
| Stickers | 52 | 38 | 52 |

[a]Number of animals.

TABLE 4. SETTLEMENT INHIBITION BY SEP-PAK FRACTIONATED LMW

| Treatment | Set | Not set | Set (%) | $G^a$ | $P$ |
|---|---|---|---|---|---|
| Control | 70 | 116 | 38 | | |
| Effluent | | | | | |
| 32 mg/ml | 155 | 276 | 36 | 00.09 | NS |
| 8 mg/ml | 160 | 174 | 48 | 04.73 | $<0.050$ |
| 80% methanol eluate | | | | | |
| 16 mg/ml | 3 | 232 | 01 | 106.87 | $<0.001$ |
| 8 mg/ml | 1 | 200 | 01 | 105.36 | $<0.001$ |
| 4 mg/ml | 14 | 234 | 06 | 70.15 | $<0.001$ |
| 2 mg/ml | 22 | 240 | 08 | 55.61 | $<0.001$ |

[a]Comparisons are made to the control. $EC_{50} = 0.600$ mg/ml (95% confidence limits 1.13–0.12 mg/ml.

ment over control. The 80% methanol eluate significantly inhibited settlement at concentrations of 16, 8, 4, and 2 mg original/ml.

Tests were made to determine if further purification of settlement inhibitor could be achieved by a stepwise elution of the $C_{18}$ cartridge. Inhibitor was applied to the cartridge in distilled water, washed with 10 ml of distilled water, and then eluted with 10 ml each of 10%, 30%, 50%, and 80% methanol. Settlement inhibitor eluted in the 80% fraction. Biological activity in the 80% eluate was comparable to that in the starting material. The recovery of solids in this fraction ranged from 6.3 to 1.0 $\mu$g/g of original homogenate.

*TLC Separation of Inhibitor*

Microscope slide silica TLC plates were developed with 95% chloroform–5% methanol. The inhibitor fraction from the Sep-Pak was separated into eight quenching zones (visible under UV light) with $R_f$ values from 0.0 to 0.9. Only the zone with an $R_f$ of 0.65–0.75 was effective in inhibiting larval barnacle settlement. Analytical reverse-phase TLC developed with 70% methanol yielded several zones with $R_f$ values of 0. to 0.5. The zone with an $R_f$ of 0.34 inhibited settlement. This zone, when chromatographed on silica in 95% chloroform–5% methanol, showed a single zone with an $R_f$ between 0.65 and 0.75.

*Preparative Scale Purification*

Procedures for obtaining settlement inhibitor were modified in order to decrease time taken to generate partially purified inhibitor and to increase the quantity of inhibitor obtained. The dialysis step and $C_{18}$ Sep-Pak step were omit-

ted and a methylene chloride extraction step and a preparative silica TLC step added. Analytical TLC on silica of material generated by the shortened extraction procedure showed an increase in organic contamination with three zones in the region of the original settlement inhibition activity. When assayed, the combined zones had an $EC_{50}$ of 2.6 ± 1.0 mg/ml. The methylene chloride extracted material gave the same general pattern on preparative silica TLC plates as was observed with analytical TLC. Mobility of high $R_f$ zones was reduced approximately 10% on preparative plates. Two of the three zones in the inhibition region inhibited settlement (Figure 1), the most potent zone, LV1 had an $EC_{50}$ of 12 ± 5 mg/ml. On a dry-weight basis, the $EC_{50}$ of LVI was approximately 0.05 μg/ml and LV2 had an $EC_{50}$ of 0.20 μg/ml.

*Affinity of Settlement Inhibitor for Polystyrene and Glass Surfaces.* The behavior of settlement inhibitor on silica and reverse-phase TLC suggests that settlement inhibitor should have markedly different affinities for polystyrene and

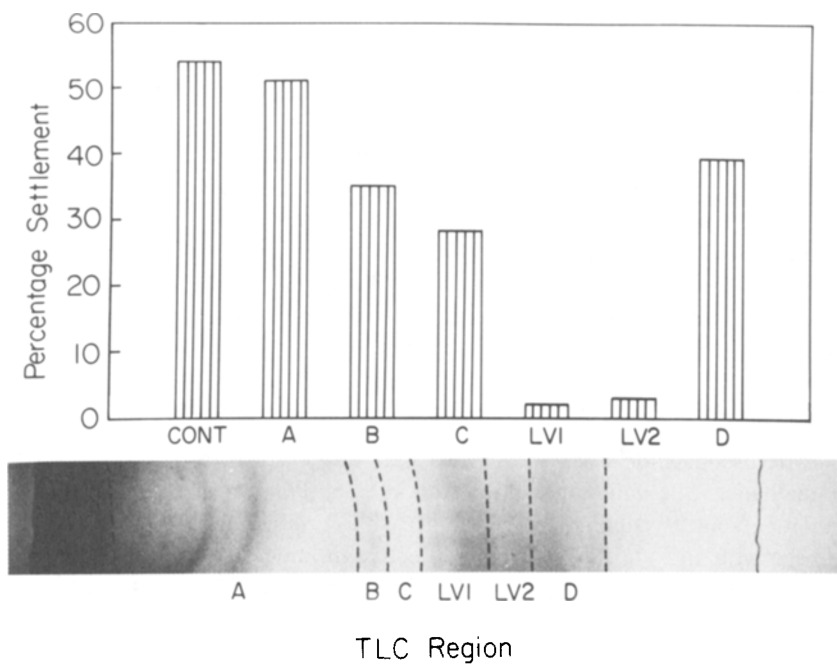


FIG. 1. Separation of *L. virgulata* settlement inhibitor by preparative thin-layer chromatography. Origin to left, front is irregular line to far right. Bars depict assay of regions between dashed lines on photo. Concentration used to generate these data was 25 mg of original homogenate/ml. At lower concentrations, only LV1 and LV2 contained significant inhibitory activity.

glass. In order to test the hypothesis that inhibitor acted by adsorbing to surfaces, a series of simultaneous parallel tests were conducted to determine the effective concentration for 50% inhibition of settlement upon glass and upon polystyrene surfaces. Glass is a substrate with relatively low affinity for inhibitor as evidenced by studies with silica TLC plates. Polystryene should have a high relative affinity because its adsorbtive characteristics are similar to the reversed-phase TLC material that showed relatively high affinity for inhibitor. Inhibitor was tested at concentrations 36, 18, 9, 4.5, 2.2, and 1.1 mg original/ml to determine $EC_{50}$ of inhibitor for the two substrates. The $EC_{50}$ for glass was 12.9 (95% confidence 15.4–9.9) and 11.7 (13.8–9.6) mg of original/ml in separate determination. Simultaneous determinations for polystyrene were $EC_{50}$ 7.4 (9.4–4.4) and 7.4 (9.4–4.4) mg original/ml, respectively. The $EC_{50}$ for inhibitor with polystyrene as the settlement substrate was significantly ($P < 0.05$) lower than the $EC_{50}$ for glass.

Next, experiments determined if adsorption to surfaces was important for activity. The experiments included glass and polystyrene test containers and four treatments.

1. Control containers: seawater, cyprids, and no other additions. Controls for treatments 3 and 4 were physically manipulated as were treatments 3 and 4.
2. Settlement inhibitor: concentration series from 18 to 6 mg original/ml. These concentrations spanned the $EC_{50}$ confidence intervals of the inhibitor preparation on both substances.
3. The same inhibitor concentration series as in 2 placed in containers for 5 min, then the inhibitor solution poured out, the containers rinsed and filled with fresh seawater.
4. Containers into which the inhibitor solution from 3 was poured.

For glass, settlement in controls was 85–98%. The inhibitor concentration series (2) showed highly significant inhibition at all dilutions. The soaked, rinsed, seawater series (3) showed statistical inhibition of settlement at rinse dilutions of 18 and 9 mg original/ml. At the most effective concentration settlement was >80% of control. The series receiving inhibitor solutions used for incubation of group 3 (4) significantly inhibited settlement at all dilutions (Table 5). Settlement of group 4 was constantly lower than settlement of group 1.

For polystyrene, settlement in controls was 65–78%. The control inhibitor concentration series (2) showed significant inhibition at all dilutions. Dishes rinsed with inhibitor (group 3) inhibited settlement at all dilutions (Table 6). Group 2 was more inhibitory than either group 3 or group 4.

Comparison of the results of the adsorption experiments for the two substrates can be summarized as follows:

1. Settlement was higher in glass controls than in polystyrene controls.
2. Settlement was significantly inhibited for all dilutions tested in series 2

TABLE 5. ADSORPTION OF INDUCER ONTO GLASS

| Treatment | Inhibitor conc | Number tested | Percent settled | $G^a$ | $P$ |
|---|---|---|---|---|---|
| 1 Control for 2 | 0 | 151 | 85 | | |
| 2 Dilution series | 18 | 152 | 51 | 45 | <<0.005 |
| | 15 | 161 | 50 | 44 | <<0.005 |
| | 12 | 167 | 56 | 33 | <<0.005 |
| | 9 | 126 | 56 | 54 | <<0.005 |
| | 6 | 166 | 63 | 21 | <<0.005 |
| 1 Control for 3 | 0 | 209 | 88 | | |
| 3 Rinsed with inhibitor solution | 18 | 128 | 73 | 11 | <0.005 |
| | 15 | 139 | 86 | 0.03 | NS |
| | 12 | 147 | 88 | 0.01 | NS |
| | 9 | 152 | 79 | 4.8 | <0.05 |
| | 6 | 130 | 84 | 0.63 | NS |
| 1 Control for 4 | 0 | 299 | 89 | | |
| 4 Received inhibitor from 3 | 18 | 88 | 32 | 106 | <0.005 |
| | 15 | 95 | 38 | 92 | <0.005 |
| | 12 | 87 | 46 | 64 | <0.005 |
| | 9 | 120 | 47 | 80 | <0.005 |
| | 6 | 114 | 61 | 40 | <0.005 |

[a]Statistical comparisons are to control.

tests independent of surface. Settlement was lower on polystyrene than it was on glass.

3. The rinsed experimental series 3 showed rinsing polystryene with inhibitor was more effective at inhibiting settlement than rinsing glass with inhibitor. Glass surfaces rinsed with inhibitor solutions were either not significantly inhibitory or inhibited settlement to about 80% of the control value. Every surface in the polystyrene series significantly inhibited settlement ($P \ll 0.005$). The least effective of the polystyrene series inhibited as effectively as the most effective of the glass treatment series. The remaining four treatments inhibited settlement to less than 65% of control values. The most effective treatment inhibited settlement to 22% of control values.

4. Sufficient inhibitor remained in the solutions that were used in rinsing surfaces in series 3 to inhibit settlement in all cases.

## DISCUSSION

Exogenous biochemical control of larval settlement is a well-known and documented phenomenon (Morse, 1984). Gorgonians contain a plethora of un-

TABLE 6. ADSORPTION OF INDUCER ONTO POLYSTYRENE

| Treatment | Inhibitor conc | Number tested | Percent settled | $G^a$ | $P$ |
|---|---|---|---|---|---|
| 1 Concentration for 2 | 0 | 170 | 65 | | |
| 2 Dilution series | 18 | 192 | 15 | 101 | <0.005 |
| | 15 | 212 | 17 | 96 | <0.005 |
| | 12 | 102 | 15 | 69 | <0.005 |
| | 9 | 92 | 13 | 69 | <0.005 |
| | 6 | 42 | 17 | 32 | <0.005 |
| 1 Concentration for 3 | 0 | 149 | 76 | | |
| 3 Rinsed with inhibitor solution | 18 | 229 | 48 | 28 | <0.005 |
| | 15 | 236 | 63 | 68 | <0.005 |
| | 12 | 94 | 42 | 27 | <0.005 |
| | 9 | 72 | 17 | 74 | <0.005 |
| | 6 | 62 | 39 | 25 | <0.005 |
| 1 Concentration for 4 | 0 | 182 | 78 | | |
| 4 Received rinse from 3 | 18 | 205 | 25 | 110 | <0.005 |
| | 15 | 255 | 30 | 101 | <0.005 |
| | 12 | 89 | 26 | 59 | <0.005 |
| | 9 | 90 | 43 | 30 | <0.005 |
| | 6 | 77 | 30 | 52 | <0.005 |

[a]Statistical comparisons are to control.

usual compounds including prostaglandins, sterols, terpenes, and cembranoides (Burkholder and Burkholder, 1958; Ciereszko et al., 1960; Ciereszko, 1962; Burkholder, 1973; Perkins and Ciereszko, 1973; Bernstein et al., 1974; Weinheimer and Matson, 1975; Cimino and De Stefano, 1978; Cimino et al., 1979; Higa et al., 1981). Indeed, the wealth of knowledge of terpenoid compounds alone has been used in determining systematic relationships of various gorgonians (Gerhart, 1983). Although biological function has not been ascribed to most of these compounds, certain of the products have demonstrated toxic, antibacterial, antiinflammatory, antineoplastic, neurophysiological, or behavioral effects (Burkholder and Burkholder, 1958; Ciereszko et al., 1960; Ciereszko, 1962; Burkholder, 1973; Perkins and Ciereszko, 1973; Bernstein et al., 1974; Weinheimer and Matson, 1975; Cimino and De Stefano, 1978; Cimino et al., 1979; Kazlauskas et al., 1978; Higa et al., 1981).

The concept that secondary compounds may function as antifoulants has been proposed frequently over the last several decades but has received little attention experimentally. Jackson and Buss (1975) demonstrated specific allelopathic effects in homogenates of encrusting organisms. Bakus et al. (1983) showed extracts from gorgonians and sponges inhibited fouling by some organisms while promoting fouling by others. Standing et al. (1984) are, to our

knowledge, the first to look specifically for compounds with barnacle antifoulant properties. Their investigations provide evidence that low-molecular-weight substances with antifoulant activity could be detected in homogenates of two octocorals (*Renilla reniformis* and *Leptogorgia virgulata*). Our studies confirm and extend the observations of Standing et al. (1984) with substances from *L. virgulata.* Three distinct types of substances have been demonstrated. One type has little affinity for reverse-phase silica and affects cyprid swimming and sticking behavior. We are not sure whether this is a debilitating effect reminiscent of the effects of gorgonian cembranolides on nudibranch larvae and ciliates (Perkins and Ciereszko, 1973; Hadfield and Ciereszko, 1978) or similar to the initial effect of $\gamma$-aminobutyric acid (Morse et al., 1979) on abalone larval behavior. Larval settlement is promoted by this fraction (Table 3). The other substances do not have activity in the behavior assay, but inhibit settlement at concentrations of less than 0.1 $\mu$g/ml, have high affinity for $C_{18}$-coated silica, and apparently function by modifying surfaces.

Semi-purified settlement inhibitor can be prepared by dialysis of homogenate, adsorption of inhibitory substances of low molecular weight on a $C_{18}$ Sep-Pak, step elution with aqueous methanol, and a combination of normal and reverse-phase thin-layer chromatography steps. The same inhibitory substances can be obtained by methylene chloride extraction of methanol-soluble substances from the homogenates, followed by preparative TLC on silica and reverse-phase TLC. Biological activity in intermediate steps of the preparative procedure can be difficult to follow due to the contaminating substances, but final products from each procedure have similar biological activity and TLC behavior.

Thin-layer chromatography of the active material reveals at least two substances with settlement inhibition activities. Behavior of the inhibitory substances in a variety of TLC systems suggests that the substances are relatively nonpolar. Further purification and characterization of the active materials is under way.

From our work it is clear that the settlement inhibitor does not directly effect the behavior of cyprids. The possibility that settlement is inhibited by an interaction of the substances with the settlement substrate is supported by observation of different $EC_{50}$s with substances and by the adsorption experiments. A mechanism that works by interfering with the physiological response of cyprids has not been tested.

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# ATTRACTANCY TO *Oryzaephilus surinamensis* (L.) OF VOLATILE MATERIALS ISOLATED FROM VACUUM DISTILLATE OF HEAT-TREATED CAROBS

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**Abstract**—Vacuum distillation of heat-treated carobs gave an aqueous, colorless, sweet-smelling distillate which was tested over a wide range of concentrations and found to be highly attractive to adult *Oryzaephilus surinamensis* (L.). The materials responsible for the aroma were isolated from the distillate by saturating with sodium chloride and extracting into diethyl ether as separate acidic, neutral, and basic fractions. The extraction efficiency was checked by recombining portions of the three fractions and replacing the diethyl ether with water to give a "reconstituted distillate;" this was almost as attractive as the original distillate. Bioassay of aqueous solutions of the three separate fractions showed that the acidic was attractive, while the neutral and basic had little effect. The five major components of the acidic fraction were found to be acetic, isobutyric, *n*-butyric, 2-methylbutyric, and hexanoic (caproic) acids. Bioassay of these in aqueous solution, both separately and combined, showed that hexanoic acid was the most attractive and may be responsible for both the longer-lasting attractive effect of the carob distillate and for the effectiveness of carobs themselves used in bait bags to detect stored product insects.

**Key Words**—*Oryzaephilus surinamensis*, saw-toothed grain beetle, Coleoptera, Silvanidae, attractant, carobs, *Ceratonia siliqua*, volatiles, vacuum distillation, hexanoic (caproic) acid.

## INTRODUCTION

During storage, cereal crops are vulnerable to infestation by numerous species of insects and mites, many of which can cause serious nutritional damage and economic loss. Early detection is essential, yet these stored product pests tend



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to be elusive and their presence is often not obvious until the infestation has become well established.

Infestations undetected by conventional inspection techniques have been successfully detected by using bait bags developed at this laboratory (Pinniger, 1975). These consist of nylon mesh bags, 18 × 9 cm, filled with a mixture of equal volumes of crushed carobs, crushed groundnuts, and whole wheat grains. Over 40 species of insects and mites have been found by using bait bags (Pinniger and Wildey, 1979). As well as being effective for surveying the occurrence, distribution, and frequency of stored product insects (e.g., Loschiavo and Okumura, 1979), the bags can also be used to check the effectiveness of an insecticidal treatment (Jacobson and Pinniger, 1982) and to collect insects for testing their resistance to insecticides.

We are currently studying, in the laboratory, the attractancy of materials isolated from crushed carobs to the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.), the major grain store pest in the United Kingdom (Freeman, 1976). Previous work (O'Donnell et al., 1983), in which materials were isolated from carobs by solvent extraction, suggested that attractancy resulted from breakdown of glycerides containing γ-linolenoyl or linoleoyl residues. However, lipid degradation is often complex, and our initial study did not suggest which of many possible compounds are actually present in carobs; neither is there any relevant literature. Moreover, while there have been studies of the olfactory responses of *O. surinamensis* to volatiles from other beetles of the same species, frass, rolled oats, and brewer's yeast (Pierce et al., 1981), and to extracts from oats (Freedman et al., 1982; Mikolajczak et al., 1983, 1984), there have been no other reports on the behavioral response of any insects to carobs. Accordingly, it has been necessary to simplify the chemical complexity of the problem initially, and the work reported here studied the attractancy to *O. surinamensis* of volatile components of carobs which were separated from the involatile materials by vacuum distillation.

## METHODS AND MATERIALS

*General.* All chemical glassware was heated to 520°C for 11 hr before use. Gas chromatography (GC) was conducted using either helium or nitrogen as the carrier gas on glass columns packed with 10% Carbowax 20 M. Reagents were of AnalaR grade. The ether ($Et_2O$) was examined for purity by GC and for freedom from peroxides by the starch–iodide test.

*Carob Distillate.* Kibbled (crushed) pods from Cypriot carob trees, *Ceratonia siliqua* (L.) (Leguminosae), were heated to 70°C for 7 hr twice, this being the way in which material to be used in bait bags is treated before use to kill any insects already present. After cooling for the second time, a 300-g portion was ground to a fine powder in a Waring blender. The powder was then heated

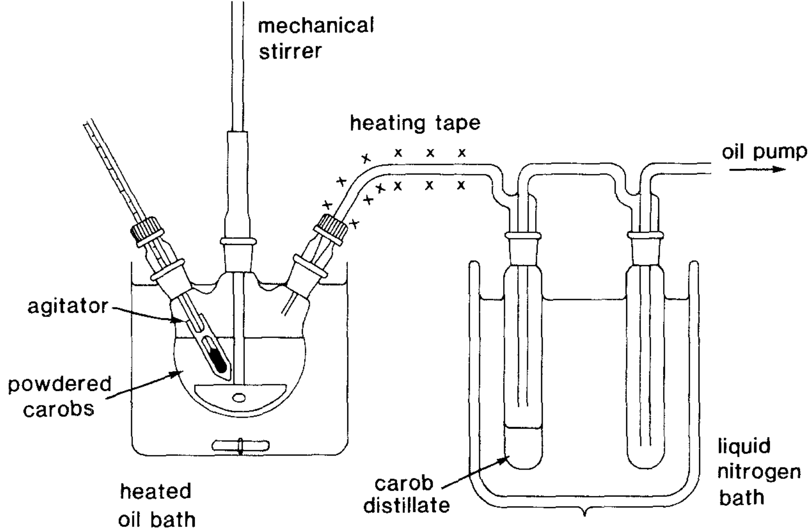


FIG. 1. All-glass vacuum distillation apparatus.

at 70°C for 3 hr under reduced pressure, 0.05 mm Hg, with mechanical agitation in the all-glass apparatus shown in Figure 1. The distillate, which collected exclusively in the first trap, weighed 23.9 g. This was freed from traces of carob powder by filtration through Whatman No. 1 paper. Three further portions of kibbled pods were then treated similarly, and the four filtered distillates were pooled. In total, 1120 g of carobs gave 81.8 g of distillate (CD), which was a colorless, sweet-smelling liquid of pH 3.15. Of several different arrangements of apparatus tested, that shown in Figure 1 gave the most reproducible results. The average yield of distillate (7.3%) was close to the presumed moisture content: when an identical batch of freshly powdered carobs was heated to constant weight in a drying oven at 70°C, the weight loss was 8.7%. Evidence that the major component of carob distillate was water included the refractive index (1.3328 at 23°C; c.f., water 1.3323) and the NMR spectrum (single signal for neat distillate at $\delta$ 4.7; c.f., water $\delta$ 4.5).

*Reconstituted Carob Distillate.* Carob distillate (60.0 g; 60.5 ml) was separated into acidic, neutral, and basic fractions in ether by the extraction scheme shown in Figure 2. The scheme was designed with the knowledge that the distillate was acidic. Ether was used as the extraction solvent since it was expected to be more effective than the commonly used dichloromethane in extracting organic acids, and acidifications were conducted with sulfuric acid since it is less soluble in ether than the more commonly used hydrochloric acid. Adjustments to pH were made slowly and with external cooling where necessary to ensure that the temperature did not exceed 25°C.

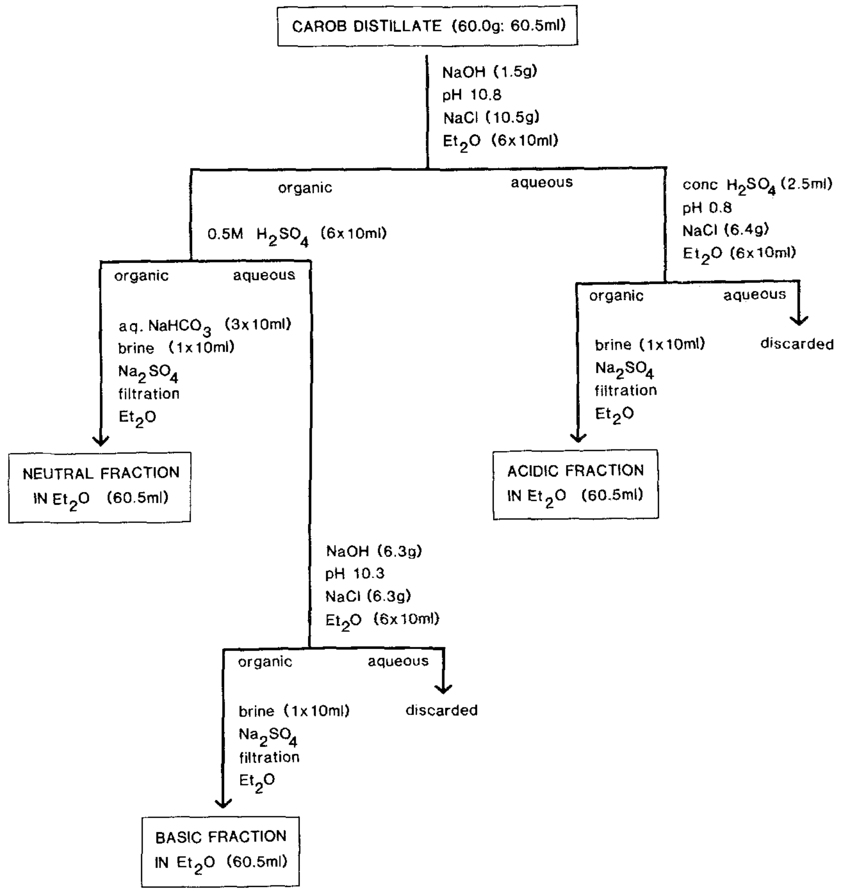


FIG. 2. Extraction scheme.

Portions (2 ml) of each of the three fractions in ether were combined. The ether was then removed by gas entrainment (MacLeod and Pieris, 1981) in the apparatus shown in Figure 3. Checks were carried out to ensure that no volatiles were carried over with the ether. Addition of 2 ml of distilled water to the residue gave RCD, an aqueous solution of reconstituted carob distillate at "original" strength. (The term "original" is used throughout this account to denote the strength equivalent to that in carob distillate itself.)

A more concentrated aqueous solution of reconstituted carob distillate was made by a similar method. Gas entrainment of a mixture of 15-ml portions of the acidic, neutral, and basic fractions in ether gave a strong-smelling yellow oil (93 mg; 110 $\mu$l) as a residue. Addition of distilled water (40 $\mu$l) and vigorous shaking gave an opaque emulsion which was used as reconstituted carob distil-

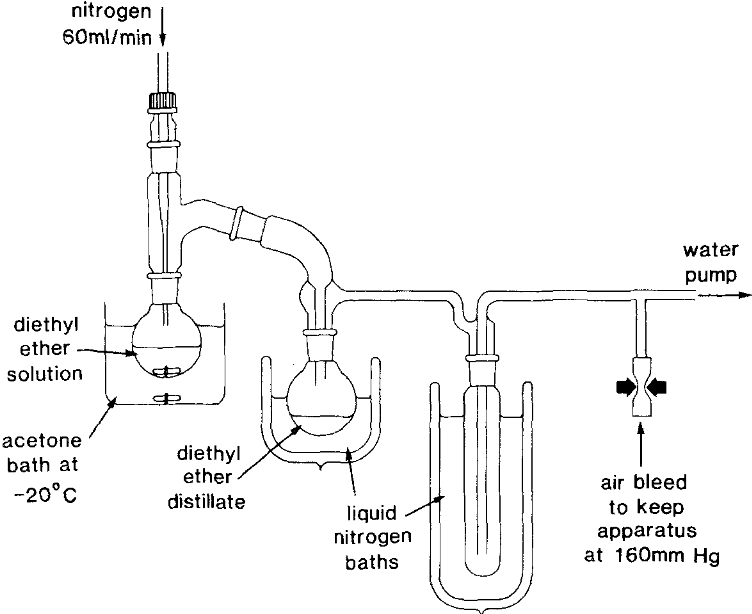


FIG. 3. Gas entrainment apparatus used to remove diethyl ether from solutions of volatiles.

late at $10^2\times$ original strength ($10^2 \times$ RCD). A portion of this emulsion was diluted with distilled water to give an aqueous solution of reconstituted carob distillate at $10\times$ original strength ($10 \times$ RCD).

*Acidic, Neutral, and Basic Solutions.* Aqueous solutions of the separate acidic, neutral, and basic fractions of carob distillate at various concentrations were made from their above individual solutions in ether by gas entrainment and the addition of water as described previously. In each case, analysis by GC confirmed that there was little loss of volatiles during the gas entrainment.

*Major Components of Fractions.* The ether solutions of the separate acidic, neutral, and basic fractions were studied by GC-MS (electron impact) and the major components were identified. The identified components were then quantified by GC by comparison with standard solutions. The solution of the acidic fraction contained acetic acid (375 ng/$\mu$l), isobutyric acid (2340 ng/$\mu$l), *n*-butyric acid (200 ng/$\mu$l), 2-methylbutyric acid (155 ng/$\mu$l), and *n*-hexanoic (caproic) acid (240 ng/$\mu$l). Close agreement was found in a comparison by GC of the aqueous solution of the acidic fraction at original strength and an aqueous solution made up to contain the five identified acids at their above concentrations. The purity of the five acids used to make up this solution was confirmed by GC, MNR, and refractive index. The solution of the neutral fraction contained 2-furaldehyde (18 ng/$\mu$l) and four unidentified components (relative total

ion currents 1.0, 2.9, 0.4, 0.3, and 2.6). The solution of the basic fraction contained 2-furaldehyde (7 ng/$\mu$l) and two unidentified components (relative total ion currents 1.0, 6.8, and 0.9).

*Preparation of Test Insects.* The insects used in the bioassays were adults taken from routine cultures of an insecticide-susceptible strain of *O. surinamensis.* The insects were cultured on a diet of whole wheat flour, rolled oats, and powdered yeast in the ratio of 5:5:1 by weight. The cultures were maintained in a darkroom at 25°C and 70% relative humidity. The insects used were of adult age 0–3 weeks and undetermined sex.

Insects were preconditioned before testing. For each replicate, 10 insects were placed in a 75 × 25-mm tube without food and kept for 24 hr at 25°C, 50% relative humidity. This had been found to be the humidity at which the maximum response to water occurred (Stubbs and Griffin, 1983).

*Bioassay Procedure.* All bioassays were carried out using apparatus based on the insect activity detector of Pinniger and Collins (1976) and described in more detail by Stubbs and Griffin (1983). Ten preconditioned insects were released into an assay arena and left for 1 hr to settle. Then, the number of crossings beneath an untreated porous polyethylene wick was recorded for two successive 5-min periods by means of a light-dependent resistor mounted in the base directly under the wick in each arena. Five microliters of test material was then applied to the wick using a microsyringe and the number of crossings measured over four consecutive 30-min periods. At the end of each assay, the insects were discarded, and all the apparatus was thoroughly cleaned. The dose–response to each group of chemicals was completed within two weeks in order to minimize the effect of any gradual change that the insects might undergo in culture.

The preapplication measurements were used to assess the general level of activity of the insects to check that there were no obvious differences between batches and thus that postapplication comparisons were valid. Preapplication scores obtained in a previous set of tests were given by Stubbs and Griffin (1983). As none of the preapplication scores in the present work appeared to differ from these, they are not presented here.

The general pattern of behavior of the insects in the arena has been described previously (Stubbs and Griffin, 1983). With a clean, dry wick, the insects spent most time at the edge of the arena. When water was applied to the wick, they moved away from the edge of the arena and towards the stimulus, thus demonstrating that the assay was one of attractancy (Kennedy, 1978). The known response to water was therefore used as a base level for comparisons in all the experiments. As all solutions tested were aqueous, water formed part of the vapor reaching the insects in all the tests. A response significantly greater than water showed that the material in solution was attractive, and one significantly lower that the material was repellent. The response may not be purely

additive, as there may be some synergistic or other interaction between the water and the material in solution.

The chemicals tested as shown in Tables 1–4: the "mixture" referred to in Table 3 contained all five of the identified acids. Dilutions were made up with distilled water. Distilled water was assayed at the same time as each group of materials to provide a control, and carob distillate at original strength, CD, was also assayed with the acids and 2-furaldehyde. Sixteen replicates were carried out for each strength of each material.

## RESULTS AND DISCUSSION

*Carob Distillate and Reconstituted Carob Distillate.* Stubbs and Griffin (1983) used batches of 10 insects to measure the response to water. Initial tests showed that these gave an accurate reflection of the response of a single insect, while simultaneously providing more data for analysis. In the present work, the same conclusion was reached for the response to various strengths of carob distillate and reconstituted carob distillate. The sex of insects was determined after the tests to avoid possible damage, and it was found that there was no difference in measured response between males and females to both carob distillate and reconstituted carob distillate, as had been previously demonstrated for water (Stubbs and Griffin, 1983).

The mean numbers of crossings under the wick $\pm$ standard errors for each time period for the various strengths of carob distillate and reconstituted carob distillate are shown in Table 1. The means are plotted in Figure 4. A Student–Newman–Kuels ranking test (Sokal and Rohlf, 1969) was carried out for each time period to detect similarities and differences in the responses to the various strengths, and the result of these tests are also shown in Table 1.

There are two main features of interest in the table of carob distillate results: the initial intensity of the response and its persistence. In the first 30-min period, carob distillate at original strength (CD), $10^{-1} \times$ CD and $10^{-2} \times$ CD all elicited a significantly greater response than the other strengths or water; the responses to $10^{-1} \times$ CD and $10^{-2} \times$ CD were significantly greater than the response to CD. After the first time period, the response diminished substantially but remained significantly greater than water for CD and $10^{-2} \times$ CD throughout the tests and for $10^{-1} \times$ CD in the second time period. The attractiveness of $10^{-1} \times$ CD seemed to decrease faster than either CD or $10^{-2} \times$ CD, but there is no obvious explanation as to why this should happen.

Original-strength reconstituted carob distillate (RCD) and $10^{-1} \times$ RCD were significantly more attractive than water in the first and second time periods. The $10^{2}\times$ and $10 \times$ RCD elicited scores significantly lower than water in the first time period. The insects appeared unsettled and moved faster than usual. When they approached the wick, they usually turned away sharply. The

TABLE 1. MEAN NUMBER OF CROSSINGS UNDER WICK ± SE FOR VARIOUS STRENGTH SOLUTIONS OF CAROB DISTILLATE (CD) AND RECONSTITUTED CAROB DISTILLATE (RCD)[a]

| Strength of solution | 1st 30-min period | 2nd 30-min period | 3rd 30-min period | 4th 30-min period |
|---|---|---|---|---|
| Carob distillate | | | | |
| Original | 71.9 ± 5.9b | 19.4 ± 3.1a | 15.1 ± 2.9a | 9.9 ± 1.5a |
| $10^{-1}$ x | 86.8 ± 5.2a | 21.5 ± 3.6a | 8.9 ± 1.7bc | 5.5 ± 1.3abc |
| $10^{-2}$ x | 90.5 ± 8.0a | 24.0 ± 5.1a | 12.8 ± 3.1ab | 10.2 ± 2.7a |
| $10^{-3}$ x | 45.4 ± 4.2c | 3.2 ± 0.5b | 1.8 ± 0.4d | 1.4 ± 0.4c |
| $10^{-4}$ x | 46.7 ± 6.0c | 4.4 ± 0.6b | 2.3 ± 0.6d | 0.9 ± 0.3c |
| Reconstituted carob distillate | | | | |
| $10^{2}$ x | 2.4 ± 1.0d | 2.7 ± 0.8b | 1.6 ± 0.3d | 2.1 ± 0.4c |
| 10 x | 9.9 ± 1.6d | 22.2 ± 3.5a | 16.1 ± 2.7a | 10.1 ± 1.5a |
| Original | 69.2 ± 5.3b | 18.1 ± 2.5a | 10.5 ± 1.9abc | 9.1 ± 1.6ab |
| $10^{-1}$ x | 70.3 ± 3.8b | 15.6 ± 1.2a | 7.4 ± 1.2bcd | 5.3 ± 1.3abc |
| $10^{-2}$ x | 51.9 ± 6.2c | 5.2 ± 0.9b | 1.6 ± 0.3d | 1.4 ± 0.3c |
| $10^{-3}$ x | 49.9 ± 5.0c | 5.4 ± 1.1b | 2.4 ± 0.5d | 1.6 ± 0.6c |
| $10^{-4}$ x | 46.4 ± 5.9c | 4.4 ± 1.1b | 2.3 ± 0.6d | 1.2 ± 0.4c |
| Water | 48.3 ± 5.0c | 7.4 ± 1.5b | 6.4 ± 1.1cd | 4.9 ± 1.4bc |

[a]Means followed by the same letter within a time period are not significantly different ($P < 0.05$).

behavior demonstrated repellency. However, by the second time period, 10 × RCD proved very attractive to the insects and remained so for the rest of the experiment. Possibly the concentration of the vapor evaporating from the wick had fallen sufficiently after 30 min to become attractive rather than repellent.

The responses to RCD and CD at original strength were indistinguishable from each other in all four time periods. However, on dilution, reconstituted carob distillate was less attractive than carob distillate itself. Some chemicals appear to have been lost during the preparation despite the precautions taken. Nevertheless, it was felt that RCD was sufficiently similar to CD to warrant further investigation. Its three components, the acidic, neutral, and basic fractions, were tested separately to determine which, if any, contained the attractive compound or compounds.

*Acidic, Neutral, and Basic Fractions.* The results are shown in Table 2 and Figure 5. In the first time period, no fraction was significantly more attractive than water at any strength tested; the $10^{2}$× and 10× concentrations were all repellent, although to varying degrees. After the first time period, none of the strengths of the basic fraction differed significantly from water. The same was

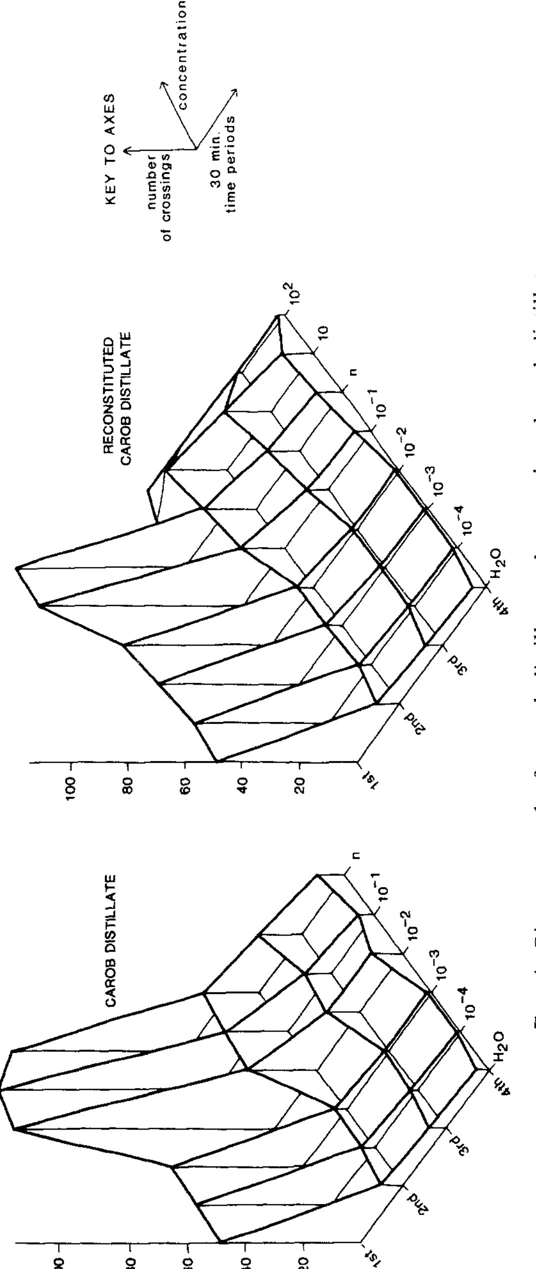


FIG. 4. Bioassay results for carob distillate and reconstituted carob distillate.

TABLE 2. MEAN NUMBER OF CROSSINGS UNDER WICK ± SE FOR VARIOUS STRENGTH SOLUTIONS OF ACIDIC, NEUTRAL, AND BASIC FRACTIONS[a]

| Strength of solution | 1st 30-min period | 2nd 30-min period | 3rd 30-min period | 4th 30-min period |
|---|---|---|---|---|
| Acidic fraction | | | | |
| $10^2$ x | 3.5 ± 1.9d | 1.2 ± 1.9d | 1.6 ± 0.7c | 1.7 ± 0.4c |
| 10 x | 4.9 ± 0.9cd | 9.1 ± 1.4c | 7.1 ± 1.3bc | 5.2 ± 1.0bc |
| Original | 56.9 ± 4.0a | 22.8 ± 2.1a | 17.2 ± 2.5a | 13.7 ± 2.8a |
| $10^{-1}$ | 50.8 ± 4.9a | 13.9 ± 2.2bc | 8.0 ± 1.5bc | 4.2 ± 1.1bc |
| Neutral fraction | | | | |
| $10^2$ x | 11.6 ± 1.7cd | 10.1 ± 1.7c | 6.1 ± 0.9bc | 6.7 ± 1.2bc |
| 10x | 28.3 ± 2.8b | 18.6 ± 2.4ab | 10.8 ± 2.2b | 8.9 ± 1.6b |
| Original | 48.9 ± 4.9a | 11.6 ± 2.0c | 5.8 ± 1.3bc | 4.1 ± 1.2bc |
| $10^{-1}$ x | 42.6 ± 5.0a | 11.6 ± 2.4c | 5.6 ± 1.1bc | 5.4 ± 1.3bc |
| Basic fraction | | | | |
| $10^2$ x | 7.4 ± 1.0cd | 5.4 ± 1.0cd | 4.1 ± 0.8c | 4.4 ± 1.1bc |
| 10 x | 19.9 ± 2.4bc | 7.7 ± 1.2cd | 2.4 ± 0.5c | 3.1 ± 0.9bc |
| Original | 54.0 ± 6.3a | 11.8 ± 2.4c | 6.3 ± 1.6bc | 3.7 ± 1.2bc |
| $10^{-1}$ x | 44.3 ± 8.9a | 10.3 ± 2.3c | 4.3 ± 1.2c | 3.3 ± 0.8bc |
| Water | 50.6 ± 6.2a | 8.4 ± 2.7c | 4.7 ± 1.6bc | 2.7 ± 1.0c |

[a]Means followed by the same letter within a time period are not significantly different ($P < 0.05$).

true for all strengths of the neutral fraction with the exception of the 10× neutral which was significantly more attractive than water in the second and fourth time periods.

In the second time period, of all the strengths of acidic fraction tested, the original strength alone was significantly more attractive than water and it continued to be so throughout the rest of the experiment. Moreover, the numbers of crossings recorded in the last three periods were similar to those obtained with original strength CD. The acidic fraction therefore contained some chemical or chemicals responsible for longer-lasting attractancy. The fact that the neutral fraction was attractive when concentrated is also of interest, suggesting that it too contained an attractive chemical in small quantities.

Therefore further study was undertaken of the five main components of the acidic fraction, acetic, isobutyric, *n*-butyric, 2-methylbutyric, and hexanoic acids, and also of 2-furaldehyde, the only compound identified from the neutral fraction.

*Five Acids and their Synthetic Mixture.* The results are given in Table 3 and Figure 6. The response to water was unexpectedly high for the first time

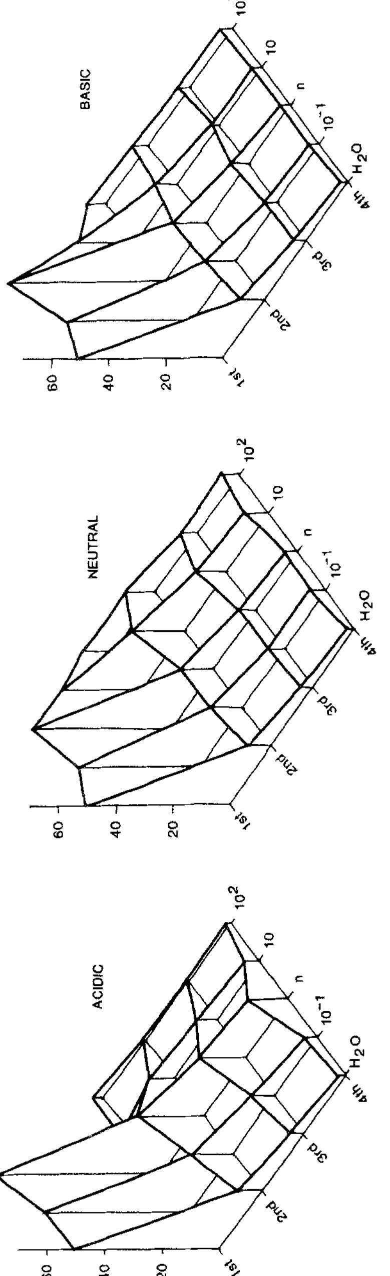


FIG. 5. Bioassay results for the acidic, neutral, and basic fractions of carob distillate.

TABLE 3. MEAN NUMBER OF CROSSINGS UNDER WICK ± SE FOR VARIOUS STRENGTH SOLUTIONS OF FIVE ACIDS AND THEIR MIXTURE[a]

| Strength of solution | 1st 30-min period | 2nd 30-min period | 3rd 30-min period | 4th 30-min period |
|---|---|---|---|---|
| Acetic acid | | | | |
| $10^2$ x | 20.3 ± 2.3de | 4.0 ± 0.9ef | 4.2 ± 0.9defg | 5.2 ± 1.7bcde |
| 10 x | 46.1 ± 6.0abc | 6.7 ± 1.2cdef | 3.9 ± 1.0defg | 3.6 ± 1.5bcde |
| Original | 49.4 ± 4.5abc | 14.0 ± 3.2abcde | 4.7 ± 0.9defg | 4.7 ± 1.6bcde |
| $10^{-1}$ x | 44.3 ± 3.7abc | 8.3 ± 1.6cdef | 4.7 ± 1.1defg | 2.7 ± 0.9bcde |
| Isobutyric acid | | | | |
| $10^2$ x | 3.6 ± 0.5e | 1.7 ± 0.5f | 1.7 ± 0.4g | 1.4 ± 0.4e |
| 10 x | 13.5 ± 1.8de | 5.1 ± 0.8def | 2.2 ± 0.6fg | 2.3 ± 0.6cde |
| Original | 63.3 ± 6.8abc | 11.0 ± 2.6bcdef | 7.9 ± 2.3cdefg | 4.6 ± 1.2bcde |
| $10^{-1}$ x | 48.4 ± 4.6abc | 6.7 ± 1.5cdef | 5.2 ± 1.0defg | 1.8 ± 0.4de |
| n-Butyric acid | | | | |
| $10^2$ x | 22.4 ± 2.7d | 9.6 ± 4.1cdef | 5.0 ± 1.1defg | 5.7 ± 1.8bcde |
| 10 x | 65.4 ± 6.5abc | 14.3 ± 1.5abcde | 5.2 ± 0.8defg | 6.6 ± 0.9bcde |
| Original | 51.8 ± 5.9abc | 5.2 ± 1.1def | 3.1 ± 0.9efg | 3.2 ± 0.5bcde |
| $10^{-1}$ x | 43.9 ± 4.8abc | 7.0 ± 1.9cdef | 5.1 ± 1.5defg | 2.7 ± 0.9bcde |
| 2-Methylbutyric acid | | | | |
| $10^2$ x | 14.0 ± 1.8de | 7.3 ± 1.1cdef | 6.8 ± 1.3cdefg | 5.2 ± 1.5bcde |
| 10 x | 66.3 ± 5.7ab | 14.7 ± 2.8abcde | 7.3 ± 1.5cdefg | 3.8 ± 1.0bcde |
| Original | 56.9 ± 5.7abc | 11.4 ± 2.4bcdef | 4.7 ± 1.4defg | 4.2 ± 1.1bcde |
| $10^{-1}$ x | 43.7 ± 6.2abc | 8.0 ± 2.0cdef | 4.8 ± 0.9defg | 2.7 ± 0.5bcde |
| Hexanoic acid | | | | |
| $10^2$ x | 10.8 ± 1.4de | 11.6 ± 1.5cdef | 11.2 ± 2.1abcd | 8.5 ± 1.6bc |
| 10 x | 42.2 ± 3.4c | 19.0 ± 3.0ab | 10.4 ± 1.8bcde | 8.1 ± 1.5bcd |
| Original | 42.9 ± 6.8bc | 22.7 ± 3.8a | 14.8 ± 3.0ab | 12.1 ± 2.3a |
| $10^{-1}$ x | 52.7 ± 4.0abc | 17.7 ± 2.9abc | 13.1 ± 2.5abc | 8.9 ± 1.9ab |
| Mixture | | | | |
| $10^2$ x | 2.3 ± 0.5e | 1.1 ± 0.3f | 1.4 ± 0.4g | 0.6 ± 0.2e |
| 10 x | 16.3 ± 1.9de | 13.6 ± 1.6abcde | 9.0 ± 1.2bcdefg | 6.9 ± 1.1bcde |
| Original | 56.6 ± 6.7abc | 16.9 ± 2.6abc | 9.7 ± 2.4bcdef | 6.2 ± 1.6bcde |
| $10^{-1}$ x | 49.6 ± 4.1abc | 15.8 ± 2.3abcd | 7.5 ± 1.3cdefg | 3.7 ± 0.9bcd |
| Water | 67.1 ± 7.4a | 9.7 ± 2.1cdef | 6.0 ± 1.6defg | 3.6 ± 0.9bcde |
| CD | 59.6 ± 9.4abc | 23.4 ± 4.2a | 16.6 ± 2.9a | 13.5 ± 2.5a |

[a]Means followed by the same letter within a time period are not significantly different ($P < 0.05$).

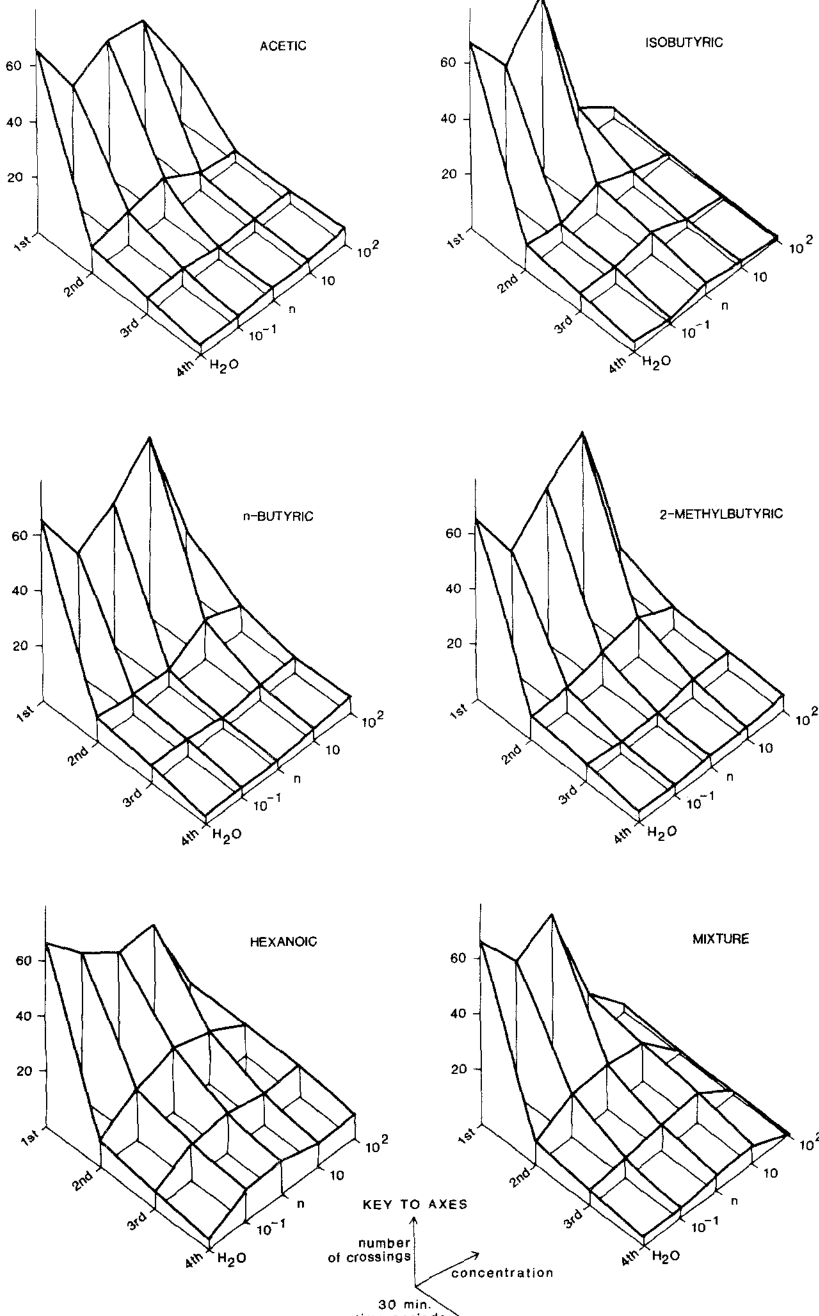


FIG. 6. Bioassay results for the five acids identified in the acidic fraction and their mixture.

period and that for CD unexpectedly low (see, for example, Table 1). There is no obvious explanation for this, as the preapplication scores were normal. After the first time period, CD and water gave very similar numbers of crossings to those in Table 1.

The responses to acetic, isobutyric, *n*-butyric, and 2-methylbutyric acids were similar. None of their solutions was significantly different from water except during the first time period, when all of the $10^2\times$ concentrations, together with the $10\times$ concentration of isobutyric acid, were repellent.

The original, $10\times$, and particularly the $10^2\times$ strengths of hexanoic (caproic) acid were significantly less attractive than water for the first time period. From the second time period onwards however, the original strength of hexanoic acid was significantly more attractive than water and not significantly different from carob distillate.

At the higher concentrations, the mixture of the five acids reflected the repellent effect of isobutyric acid, while at original and $10^{-1}\times$ strengths in the second time period it reflected the attractive effect of hexanoic acid. However, unlike hexanoic acid alone, the mixture did not emulate the lasting attractancy of the acidic fraction at original strength. The mixture contained only the five identified acids. Anything present in small quantities in the acidic fraction would be absent from the mixture. The absence of these trace materials may explain why the mixture was not as attractive as the acidic fraction or CD.

*2-Furaldehyde.* None of the solutions of 2-furaldehyde was significantly different from water at any period (Table 4). However, the $10\times$ strength in the second time period was not significantly different from CD. This suggests that it was slightly attractive and may have been partly responsible for the attractancy of the $10\times$ neutral fraction in the second time period (Table 2). Mikolajczak et al. (1984) have recently reported that similar doses of 2-fural-

TABLE 4. MEAN NUMBER OF CROSSINGS UNDER WICK ± SE FOR VARIOUS STRENGTH SOLUTIONS OF 2-FURALDEHYDE[a]

| Strength of solution | 1st 30-min period | 2nd 30-min period | 3rd 30-min period | 4th 30-min period |
|---|---|---|---|---|
| 2-Furaldehyde | | | | |
| $10^2$ x | 33.7 ± 3.7b | 4.2 ± 0.8b | 2.6 ± 0.5b | 2.9 ± 0.7b |
| 10 x | 48.9 ± 7.0ab | 9.7 ± 2.6ab | 5.5 ± 1.8b | 3.6 ± 1.1b |
| Original | 52.0 ± 3.4ab | 5.4 ± 1.1b | 4.0 ± 0.8b | 3.4 ± 0.7b |
| $10^{-1}$ x | 42.4 ± 5.1ab | 5.1 ± 1.0b | 3.8 ± 0.8b | 2.6 ± 0.5b |
| Water | 40.2 ± 5.0ab | 5.2 ± 0.8b | 3.5 ± 0.8b | 2.8 ± 0.5b |
| CD | 57.2 ± 4.0a | 14.0 ± 2.3a | 11.4 ± 1.9a | 8.7 ± 1.2a |

[a]Means followed by the same letter within a time period are not significantly different ($P < 0.05$).

dehyde are attractive in a pitfall bioassay to adults of *O. surinamensis* between 4 and 6 weeks old.

*General Discussion.* For all materials examined in the current work, the maximum attractive response was achieved within the range of strengths tested. Therefore there was no need to test either more concentrated or more dilute solutions.

Of the individual chemicals tested, hexanoic acid has been found to be the most attractive to *O. surinamensis.* This result agrees well with previous findings (O'Donnell et al., 1983) that the attractancy to *O. surinamensis* of solvent extracts of carobs is probably related to glyceride breakdown: the most effective triglycerides contained $\gamma$-linolenoyl or linoleoyl residues, and these would give hexanoic acid as the most abundant volatile product of oxidative degradation. Levinson and Kanaujia (1981) reported that the arrestant effect of wheat on *Sitophilus granarius* (L.) may similarly be due to the formation of fatty acids during storage, but the acids were of longer carbon chain than those tested in the current study.

In a review of the effect on some stored product insects, Levinson and Levinson (1978) quote that these higher fatty acids (above $C_{12}$) are, in general, either aggregants or phagostimulants, whereas the lower fatty acids (including hexanoic acid) are repellent. For example, Cohen et al. (1974) reported that hexanoic acid repels adults of *Trogoderma granarium* (Everts) and *Tribolium castaneum* (Herbst) and larvae of *Dermestes maculatus* (De Geer), but they added that it is a feeding stimulant for *D. maculatus* adults. Nevertheless, the tests were conducted over only a narrow range of doses; consequently, it is conceivable that the results might be misleading. However, Yamamoto et al. (1976) stated that hexanoic acid is the major single attractant in rice and corn for *Sitophilus zeamais* (Motsch.). Greenblatt et al. (1976) proposed that it is a releaser to copulatory behavior in adult male *T. glabrum,* and Karlson et al. (1968) claimed it to be a putative trail pheromone of the termite *Zootermopsis nevadensis* (Hagen). Other than this, there has been little behavioral work with hexanoic acid and its effect on *O. surinamensis* has not been reported previously.

Apart from our own studies, the only other behavioral work with *O. surinamenis* with any detailed mention of chemicals tested are two studies on oat constituents by Mikolajczak et al. (1983, 1984). The latter dealt with aldehydes, but a major part of their earlier study referred to the effects of acids. In general these were the higher fatty acids in either free or esterified form, but they did test a mixture of volatile $C_6$–$C_{14}$ free fatty acids in unspecified proportion. They found it to be repellent at the higher doses they used and less so at the lower doses, but it did not stimulate aggregation at any strength tested. This is not surprising in view of our own results with the free fatty acid mixture, although admittedly it is difficult to compare results obtained with such different bioassay methods. Nevertheless, it reinforces our view that the study of potential attrac-

tants in complex mixtures of natural products is often confused by the presence of repellents. In the present investigation we have studied all the identified chemicals individually. This was possible because vacuum distillation successfully removed the limited number of volatiles from the large number of involatile compounds present in carobs.

The vapor produced by carob distillate is of complex composition, and it is unlikely that its overall behavioral effect could be produced by one chemical. However, among the components of the distillate identified so far, hexanoic acid has been shown to be the most effective single compound in attracting *O. surinamensis*. It may be responsible for the longer lasting attractive effect of carob distillate and for the effectiveness of carobs themselves in bait bags, and will be used in future work on insect detection.

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# ALLELOPATHIC EFFECTS OF GIANT FOXTAIL ON GERMINATION AND RADICLE ELONGATION OF LOBLOLLY PINE SEED

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**Abstract**—Water extracts of giant foxtail grass inhibited germination and radicle elongation of loblolly pine seed in a laboratory test. Most of the toxic effects came from extracts of dried foxtail tops, with lesser amounts from fresh tops and roots. Soil texture affected the phytotoxicity of the extracts. Eight chemical compounds known to be phytotoxins in *Setaria* were identified in extracts of foxtail leaves but could not be found in soil samples under the leaves.

**Key Words**—*Pinus taeda, Setaria,* seed germination, radicle elongation, allelopathy, phytotoxins, soil texture.

## INTRODUCTION

The slow invasion of abandoned agricultural fields by loblolly pine (*Pinus taeda* L.), even when seed-producing trees completely surround the area, is of special interest to the forest manager who wishes to obtain natural loblolly regeneration in those fields. The reason given for this dearth of pine reproduction is that the seedlings could not compete for moisture, nutrients, and/or light with the vegetation already established on the area. Recently, several investigators have reported that substances produced by certain lesser vegetation inhibit seed germination and growth of conifers (Brown, 1967; Rietveld, 1975; Gilmore, 1979; Norby and Kozlowski, 1980). These reports suggest that chemical interactions (allelopathy) might play an important role in the establishment and growth of pine in these old fields.

A primary invader of old fields in the midwest is foxtail (*Setaria* sp.). These annual grasses immediately invade abandoned fields and form a dense cover.



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The establishment of other vegetation on these areas, after foxtail is established, is difficult for a few years. Gilmore and Boggess (1963) attributed the low survival and growth of planted loblolly pine in southern Illinois partly to giant foxtail (*Setaria Faberii* Herrm.) which competed severely for light and moisture. Recently, Gilmore (1980) reported that in a greenhouse experiment giant foxtail exhibited a phytotoxic effect on the growth of loblolly pine seedlings.

The inhibitory effects of foxtail on growth of young loblolly seedlings is important, but the primary concern in natural regeneration is seed germination and seedling establishment. Foxtail normally has completed its growth cycle, and the dead mat of leaves, stems, and roots are decomposing at the time pine seeds are being dispersed. If substances are released from foxtail that inhibit seed germination and radicle growth of pine, this might partly account for the erratic establishment of loblolly pine in an old field covered with foxtail. The results of a laboratory study designed to determine these effects and to identify the possible phytotoxins are reported in this paper. The study was divided into five phases.

## PHASE I

The first phase of the study was to determine whether or not roots or shoots of giant foxtail inhibit germination and/or radicle elongation of loblolly pine seed.

### *Methods*

Green mature giant foxtail plants were lifted in early August from an abandoned field with special care being taken to maintain the root system intact. Excess soil was shaken from the roots, but the plants were left unwashed to avoid losing any phytotoxins that might be present. Approximately half the plants were air dried for 40 days. The remaining plants (hereafter referred to as fresh tops or roots) were frozen until used in the experiment. Roots and tops of the dried plants were ground separately in a Wiley mill to pass a 20-mesh screen.

Water extracts of fresh tops, fresh roots, dried tops, or dried roots of giant foxtail were obtained by homogenizing plant material in a blender with distilled water for 10 min. The plant extract was filtered, washed with distilled water, and made to volume with water so that the ratio of water to plant material was 30:1 (w/w), based on samples oven dried at 70°C.

Seed used in the experiment was collected in eastern Virginia in 1975 and held in cold storage since the time of collection. Before the seed was used in the experiment, trash, wings, and empty seed were removed by floating in water. All seed were surface sterilized with 30% hydrogen peroxide for 10 min. Peroxide was removed from the seed coat by washing six times with sterilized distilled water.

Twenty-five sterilized seed were placed in each sterilized 9-cm Petri dish containing a double layer of filter paper. Either 10 ml (equivalent to 0.33 g of plant material) of one of the four plant extracts or distilled water were added to a Petri dish. This amount of material added to a dish represented 58% and 7% of the quantity of foxtail material in roots and tops, respectively, of that found in an equal-sized area in a field invaded by foxtail (field amount computed from data of Knake, 1972). Distilled water was added when additional moisture was needed. Treatments were replicated five times using a total of 25 Petri dishes.

The experiment was conducted in a room air conditioned (24°C) only during the day, but night temperatures seldom varied more than three degrees from day temperatures. No special light conditions were imposed on the experiment. The Petri dishes were arranged randomly, and the positions were changed every third day to minimize location effects.

A seed was considered to be germinated when the radicle protruded from the seed coat. Germination was recorded daily for 19 days. Radicle lengths of the first four seed to germinate in each Petri dish were measured daily throughout the experiment. Fungal contamination was not considered serious until the last day of the experiment.

Rate of germination (Maguire, 1962) (obtained by dividing the percent of germinating seed each day by the number of days seed are in the Petri dishes and then summing all values), total seed germination, and radicle elongation were tested for statistical differences by the randomized block and paired $t$ test methods.

## *Results and Discussion*

*Seed Germination.* Total germination of seed treated with distilled water (control) was 81%. Extracts of dried giant foxtail tops and roots and fresh foxtail tops affected the total percentage of seed that germinated in the experiment (Table 1). Compared to the control, 17% fewer seed germinated when they were treated with extracts from dried materials and from 1 to 13% less when treated with extracts from fresh material. A possible reason for these differences in germination between seed treated with extracts of dried and fresh plant material is either that the inhibitors were released more readily when the plant tissue and cell organization were ruptured during the drying and grinding processes or that drying converted one or more nontoxic compounds into toxic compounds.

Total percentage of seed that germinate does not necessarily give an accurate measure of the effects of an inhibitor. When the rates of seed germination were examined, it showed that germination of seed treated with distilled water (control) was faster than seed treated with extracts of fresh foxtail roots even though no statistical differences were found in total germination between the two treatments. For example, germination percentages of the control seed and those seed treated with extract from fresh foxtail roots were 63% and 50%,

TABLE 1. SEED GERMINATION AND RADICLE ELONGATION OF LOBLOLLY PINE AS AFFECTED BY EXTRACTIVES FROM GIANT FOXTAIL[a]

| Leachates from | Germination (% of control) | Elongation (total mm) |
|---|---|---|
| Dried tops | 83a | 33.6a |
| Dried roots | 83a | 51.8ab |
| Fresh tops | 87ab | 48.3ab |
| Fresh roots | 99bc | 52.4ab |
| Control (water) | 100c | 62.0b |

[a]Values in a vertical column followed by the same letter are not significantly different $\alpha = 0.05$.

respectively, at the end of 10 days, and 78% and 72%, respectively, at the end of 15 days, with total germination the same for the two treatments at the end of the experiment (19 days). Similarly, germination percentages of those seed treated with extracts of dried foxtail tops were different from those treated with extract of dried roots or fresh tops. The rate of seed germination under certain conditions could be a significant factor in natural reproduction.

Results from this phase of the experiment substantiated the results of an exploratory study which found that the greatest reduction in seed germination occurred when seed were treated with extracts of dried foxtail tops, followed in order by extracts of dried roots, fresh tops, fresh roots, and distilled water.

*Radicle Elongation*. The radicles of the control plants appeared to be longer than the radicles in the other treatments, but the only statistically significant difference was between the control and seed treated with extracts from dried foxtail tops (Table 1). Radicle elongation in the control group averaged 1.85 times those in the dried tops group. Total radicle elongation was greater but not significantly so, for seed treated with root extracts than for those treated with extracts of tops. But, as previously noted, the root extracts represent about eight times more of the actual root dry biomass than the top extracts do of the actual top dry biomass for the area of the Petri dish. These results substantiate the assumption that more inhibitory substances came from foxtail tops than from roots.

The rate of radicle elongation was more inhibited by extract from dried foxtail than from the other treatments. The total radicle elongation 10 days after the start of the test was 13 mm for seed treated with extract from dried tops and 19 mm for the control. After 15 days, elongation was 30 and 49 mm, respectively, and at the end of the experiment elongation was 34 and 62 mm, respectively. These results show that the effect of the extract became more pronounced the longer the radicle was exposed to the extract.

Results from this phase of the study do not necessarily mean that similar results will occur in the field. Seed in this study were subjected to extracts from either foxtail tops or roots, but in the field the seed and elongating radicles are exposed to compounds being released from both foxtail tops and roots. Thus, one would expect that at least as much, if not more, inhibition of seed germination and radicle elongation would occur in the field.

## PHASE II

A second phase of the study was to determine if certain phenolic compounds reported by Chou and Young (1975) to occur in *Setaria* were present in the foxtail samples used in phase I of the study. The compounds reported were *O*-hydroxyphenylacetic, vanillic, syringic, *p*-coumaric, ferulic, and caffeic acids. In addition, gentisic acid and scopoletin were added to the list of phenolic compounds to be investigated in the present study.

### *Methods, Results, and Discussion*

Compounds were extracted by homogenizing dry foxtail tops in distilled water for 10 min. The compounds were removed from the water extract with acidified acetone. The acetone was removed by freeze drying and the compounds picked up in BSTFA, after which the suspected phytotoxic compounds were determined by GC-MS. All of the eight compounds were present in the foxtail samples used in the present study. This finding does not establish that any of these compounds are phytotoxic to germinating loblolly pine seed and subsequent radicle growth, but it does indicate the possibility of inhibition due to the presence of one or more of them.

## PHASE III

It has been demonstrated in this study that water extracts of giant foxtail inhibit seed germination and radicle elongation of loblolly pine seed and that six compounds found in *Setaria* and reported to be of a phytotoxic nature are found in the giant foxtail used in this study. The next phase of the study was to determine if water leaching directly from foxtail leaves onto loblolly pine seed would inhibit seed germination when seed were in contact with soil of a sandy or silt loam texture.

### *Methods*

Petri dishes were half filled with either a sandy or a silt loam topsoil on which were placed 50 loblolly pine seed. On top of the seed in a dish was placed one of four levels of cut-up dried foxtail leaves (0.3, 0.6, 1.2, or 0.0 g/dish)

replicated four times. The seed and soil were kept moist by spraying distilled water on the foxtail leaves in each dish. Seed germination was determined for 30 days and tabulated by soil texture, foxtail treatment, and replication.

*Results and Discussion*

Germination of the control group of seed was 91% for both soil textures at the end of the test period. There was no difference in percent seed germination between the three foxtail treatments for each soil texture, but these treatments were different from the control (Table 2). These results indicate that a small amount of a toxic compound can inhibit loblolly pine seed germination if the compound is being constantly replenished (that is, leaching from the leaves onto the seed) and concentrated in the soil.

Sand did not affect seed germination, whereas there was about a 25% reduction in germination on a silt loam soil. This suggests that phytotoxins were not concentrated in the sand because of leaching of the compounds or that due to better aeration of the sand, oxidation of the compounds was more rapid. On the other hand, the silt loam soil was not as well drained or aerated and the compounds more than likely accumulated to toxic levels near the surface of the soil sample because of less oxidation and more bonding sites on the soil micelle. This hypothesis is supported by Fisher's (1978) study between black walnut and mixtures of red and white pines. He found that on imperfectly and poorly drained fine sandy loam soils, black walnut suppressed or killed the pine. In a laboratory test he found that the inhibitory activity of juglone readily disappeared from soil under a "dry moisture regime" but remained in the soil under a "wet moisture regime."

A number of reports in the literature (Lerner and Evenari, 1961) have ad-

TABLE 2. MEAN VALUES OF SEED GERMINATION ACCORDING TO TREATMENTS AND SOIL TYPES

| | Percent of control[a] | | | |
|---|---|---|---|---|
| | Grams foxtail per dish | | | |
| Soil texture | 0.3 | 0.6 | 1.2 | Mean |
| Sand | 96 | 98 | 97 | 97 |
| Silt loam | 74 | 74 | 79 | 76 |
| Mean[b] | 85 | 86 | 88 | |

[a]Mean values for sand are significantly different ($\alpha = 0.01$) from silt loam values for all treatment.
[b]Means of all treatments are significantly different ($\alpha = 0.01$) from the control.

vised caution in extending laboratory data which show allelopathic effects to ecological significance in the field as final proof is obtained only under field conditions. However, laboratory results obtained in this study should extend to field conditions because, in a field occupied by giant foxtail in the fall of the year, pine seed are normally on top of the soil under a mat of dead leaves and stems, subject to rain and dew flowing over and through the dead vegetation. This water should be high in compounds leached from the foxtail when it comes into contact with the seed and soil. As shown in this study, chemical compounds in the dead foxtail leaves are those that affect seed germination and radicle elongation. However, germination and radicle elongation of seed over a fine textured soil could also be affected by compounds that had been changed by chemical conversion or soil microorganisms into phytotoxins.

## PHASE IV

The next phase of the study was to determine if these six known phytotoxins plus gentisic acid and scopoletin found in the foxtail leaves were present in the soil from phase III of the study.

### *Methods, Results, and Discussion*

The soil samples in the Petri dishes from phase III were air dried and 50 g were collected from the top of each sample. The replications were combined into eight samples representing the four treatments and two soil types. The soil samples were ground to pass a 1-mm sieve and a 30-g sample was taken from each of the eight samples. These samples were extracted with 50 ml of water for 2 h in a reciprocating shaker. The resulting solutions were extracted three times with 10-ml volumes of dichloromethane ($CH_2Cl_2$). Investigations in our laboratory have shown that $CH_2Cl_2$ extracted similar compounds from water extracts of giant foxtail as did acidified acetone and was an easier procedure to use. The $CH_2Cl_2$ was evaporated, 0.5 ml of BSTFA was added to each vial, and compounds were determined by GC-MS analyses. A large number of chemical compounds was found in the samples, but none of the eight phenolic compounds found in the extracts from foxtail leaves was present.

It is impossible to state, with the information available, whether the eight compounds were leached out of the soil, fixed on soil micelles, degraded, or converted into nontoxic compounds. Because of the short time involved in the study, a guess is that these eight compounds had been fixed on the soil matrix. Katase (1981) reported a scheme showing the relative activity of four of the six phytotoxins found in *Setaria* in a forest soil that should relate to our experimental conditions. The free form (A) is the most easily extracted and is the most active in inhibiting growth of plants; a second form (B) is less easy to extract

from the soil and occurs as a combined form that has little inhibitory power to plants in its existing form; and the third form (C) is bound to the soil matrix and does not move easily in the soil environment. He reported that the relative concentrations of one of these compounds (vanillic acid) in a forest soil was 3, 5, and 92% for forms A, B, and C, respectively. It is suggested that the phytotoxins as leached from the dead foxtail in our study were mainly form A and if any of the phytotoxins remained in the soil it was form C.

## PHASE V

There are conflicting reports in the literature as to the correct solvent to use in extracting phytotoxic compounds from the soil. These extractants include, among others, bases, acids, organic solvents, and water. In one study using water as the extracting solvent, Whitehead et al. (1982) reported that they determined a number of phenolic compounds from the soil that are known to be phytotoxins, but these compounds were of insufficient quantities to exert an allelopathic effect. As reported in phase IV of this study, none of the eight phenolic compounds could be detected in the soil-water extracts, but this does not necessarily mean that all of these compounds were in insufficient quantities to inhibit seed germination. The best indicator of germination inhibitors is to use the seed in question. To determine if germination inhibitors of seed were present in the soil, seed were germinated on samples of soil from phase IV of the study.

### *Methods, Results, and Discussion*

A 50-g sample of soil from each treatment was placed in individual Petri dishes, and 15 loblolly pine seed were placed on top of the soil. The soil and seed were kept moist with water and germination was checked for 40 days.

There was no difference in seed germination that could be attributed to past treatment. Thus, the phytotoxins that were present in the water extracts from foxtail leaves were not active in the soil samples. This indicates that if the compounds had been present in the soil, a rapid chemical conversion had taken place or else the compounds were being held tightly on soil micelle which rendered the original phytotoxins nontoxic.

## CONCLUSIONS

Seed germination is important in natural regeneration, but this study shows that more than an adequate number of seed will germinate even when they are exposed to water extracts of giant foxtail; thus, a fully stocked stand should be obtained. The critical factor in natural reproduction, however, is seedling survival. For a seedling to survive, it must establish a good fibrous root system

early, one that will enable it to compete with other vegetation for moisture. Hindrance to early growth of the root system could be very critical in droughty areas or on those sites that are subject to periodic droughts.

As shown in the study, there are one or more compound(s) in giant foxtail that can impede loblolly pine seed germination and radicle elongation, but there does not appear to be a carryover of any of the suspected phytotoxins in the soil. It is assumed that the inhibition is from direct absorption of substance(s) mainly from tops leachate and not from soil accumulation. It is unlikely that the roots contribute much, if any, of the inhibitor(s) to seeds that germinate on the soil surface. Therefore, it is safe to assume that if the source of these phytotoxins were eliminated, the persistence of the toxins would be of limited duration. If natural regeneration of loblolly pine is desired in an old field overgrown with giant foxtail, or another primary plant invader that is thought to inhibit pine seed germination and seedling growth, it might be desirable to control-burn the area before the pine seed have been dispersed. If seed dispersal takes place before the area can be burned, it might be advisable to run a "cool" fire through the area so that the pine seed on the ground are not unduly harmed. The burn in either instance will more than likely destroy the vegetation that contains the suspected phytotoxins and help to ensure a higher germination percentage and a denser stand of pine seedlings. As has been demonstrated in numerous studies (Rice, 1974), even a temporary slowing of germination or seedling growth can affect the balance in ordinary competition among plants in a community and favor those plants that develop rapidly.

This study also shows that phytotoxins in giant foxtail are rapidly converted to nontoxic substance(s) in the soil, which are the traits looked for in weed science. Additional research should be directed toward finding pesticides with these desired properties that are effective under different edaphic and climatic conditions.

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# CHEMICAL INVESTIGATIONS OF WOLF (*Canis lupus*) ANAL-SAC SECRETION IN RELATION TO BREEDING SEASON

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**Abstract**—The volatile constituents of wolf anal-sac secretions were examined via capillary gas chromatography and compared among intact males, females, castrate males, ovariectomized females, and anosmic and pinealectomized males and females. Some chemical compounds were deemed significantly different (*t* test, 95% confidence level) among the groups both during and outside of the mating season, implying that the volatile components of anal-sac secretion can be used to communicate information regarding gender or endocrine state. As a result of treating the anal sac with antibiotics, some of these compounds, including 1-octen-3-ol and indole, were implicated as being products of microbial action. In addition, short-chain carboxylic acids were investigated and essentially no significant variations were seen among the groups.

**Key Words**—Wolf anal-sac secretion, volatile components, gas chromatography, carboxylic acids.

## INTRODUCTION

The majority of mammals possess glands that produce secretions used in scent marking. These secretions are deposited onto objects by rubbing or, in the case of scent glands near the anus, by defecation (Johnson, 1973; Asa et al., 1984).



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Guinea pigs (*Cavia porcellus*) use the perineal gland to deposit scent by moving their perineal regions across surfaces, and adult males can distinguish the odor of their own gland material from that of others (Beruter et al., 1974). The major components of the odor were short-chain aliphatic acids. Acids are commonly seen in anal-gland secretions (Goodrich et al., 1981; Albone et al., 1974; Preti et al., 1976; Doty and Dunbar, 1974; Gorman et al., 1974; Albone and Perry, 1975; Sokolov et al., 1980; Peters, 1974). A list of acids seen in anal-sac secretions of various mammals is presented in Table 5. Volatile fatty acids have also been found in a variety of other samples, including the vaginal secretions of the rhesus monkey (*Macaca mulatta*) (Michael et al., 1972) and humans (Michael et al., 1974).

Rabbits (*Oryctolagus cuniculus*) deposit secretions from their anal glands onto feces during defecation (Goodrich et al., 1981). Various scent components in rabbit secretions include hydrocarbons, alcohols, ketones, esters, and carboxylic acids. Although the structures of anal glands of rabbits are different from those of canids, they are mentioned here (and in Table 1) because of the types of compounds found. Such compounds, especially those with functional groups, are generally considered odoriferous. This property could provide for a more efficient message communication. Examples exist in the literature for both insects and mammals (Ritter, 1979; Shorey, 1976).

Three functions have been suggested for anal-sac secretions in the canids: (1) sexual attraction (Albone et al, 1974; Preti et al., 1976), (2) individual recognition and territorial demarcation (Albone et al., 1974; Preti et al., 1976; Peters and Mech, 1975), and (3) alarm function (Albone et al., 1974; Preti et al., 1976; Albone and Fox, 1971; Doty and Dunbar, 1974). Doty and Dunbar (1974), utilizing beagles, found no support for the sexual attraction hypothesis; however, they did see evidence for an alarm function, because some males in one of their experiments howled and pawed at the floor of the test apparatus apparently in response to anal-sac secretion.

The present study was undertaken to characterize the volatile components of wolf anal-sac secretion utilizing preconcentration and derivatization gas chromatographic methodologies. Information extracted from these chromatographic data allowed qualitative and semiquantitative comparisons among male, female, castrate male, etc., to see whether the animals could use such information for communication. Based on the facts that each wolf has a right and left anal gland and that the animals have voluntary control over expulsion (Asa et al., 1984), any differences in the volatile composition, i.e., left vs. right, could convey different messages. This possibility was also examined.

## METHODS AND MATERIALS

*Samples.* Anal-sac samples, obtained by syringe from anesthetized animals, were shipped in dry ice to Bloomington, Indiana, from a wolf colony

TABLE 1. ACIDS FOUND IN ANAL-GLAND SECRETIONS OF VARIOUS MAMMALS

| Acid | Wolf (Peters, 1974) | Wolf (present study) | Fox (Albone and Fox, 1971; Albone et al., 1974) | Lion (Albone et al., 1974) | Mongoose (Gorman et al., 1974) | Dog/coyote (Preti et al., 1976) | Mink (Sokolov et al., 1980) | Rabbit (Goodrich et al., 1981) |
|---|---|---|---|---|---|---|---|---|
| Acetic | √ | √ | √ | √ | √ | √ | √ | √[b] |
| Propanoic | √ | √ | √ | √ | √ | √ | √ | √[b] |
| 2-Methylpropanoic | √ | √ | √ | √ | √ | √ | √ | |
| *n*-Butanoic | √ | √ | √ | √ | √ | √ | | √[b] |
| 3-Methylbutanoic | | √ | √ | √ | √ | √ | | |
| 2-Methylbutanoic | | √ | √ | √ | | √ | | |
| Pentanoic | √ | | √ | √ | √ | √ | √ | |
| 2-Methylpentanoic | | | | | | √ | | |
| 3-Methylpentanoic | | | | | | | √ | |
| 4-Methylpentanoic | | | √ | √ | | √ | √ | |
| Benzoic | | √ | | | | | | |
| Phenylacetic | | | √[a] | √[a] | | | | |
| 3-Phenylpropanoic | | | √[a] | √[a] | | | | |
| *p*-Hydroxyphenylacetic | | | √[a] | √[a] | | | | |
| 3-(*p*-hydroxyphenyl)-propanoic | | | √[a] | √[a] | | | | |

[a]As methyl esters or trimethylsilyl ethers (BSA).
[b]Induced heart rate change in male rabbit.

maintained near Minneapolis, in acid-washed vials with Teflon-lined caps. The samples were cataloged and kept frozen until used.

The samples examined in this work were taken from animals in February and August 1981. These months were chosen to maximize the probability of elucidating any gender-related differences in the volatile components during the breeding season (January–March) versus other months. Serum levels of sex steroid hormones are at their nadir during the summer and peak during winter (Seal et al., 1979). In addition to secretions from intact males and females, samples from ovariectomized females, castrate males, and anosmic and pinealectomized males and females were analyzed to determine whether their altered states changed the volatile composition of their anal-sac secretion. An anosmic animal may be at a disadvantage if there are primer or releaser effects that require the sense of smell, and the pineal gland influences the functioning of the gonads (Oksche, 1980).

Before any analyses were carried out, the samples were diluted with clean, distilled water to facilitate sample handling. This treatment does not affect the resulting chromatograms, as determined by comparing the volatile profile obtained from an aliquot of secretion sampled directly with that obtained from an aliquot of secretion sampled after dilution with water. Aliquots of samples from the same data and sample group were pooled to minimize individual differences. This procedure should yield chromatographic profiles more representative of each group. For intact males and females, secretions from two to five animals were combined. In general, the secretions from surgically altered animals were not pooled due to the small number of such animals. Ultimately then, for each group, there were two to four samples from February and two from August, the larger number representing samples from intact animals. However, there were no samples from anosmic animals in August (Table 2).

*Chromatographic Investigations of Volatiles.* The chromatographic profiles, resulting from the separation of the volatile components of a sample, were obtained via methods of Novotny et al. (1974). Peak areas resulting from replicate analyses of the same samples indicate a maximum of 15% variation with less than 10% being typical (J. Raymer, unpublished observation). Headspace sampling was accomplished by purging with purified helium (100 ml/min for 1 hr) 1 ml of sample (typically about 0.3 ml diluted with 1 ml $H_2O$) to which 0.3 g $(NH_4)_2SO_4$ had been added. The sample was thermostated at 50°C during sampling. The vapor was then passed through a chilled condenser before encountering 4.0 mg Tenax GC in a glass precolumn.

Subsequent gas-chromatographic (GC) analysis, using a Perkin-Elmer model 3920 GC with a modified sampling port and detector, of the anal-sac secretion volatiles was carried out with glass capillary columns (Novotny et al., 1974). The column used in this work was a glass capillary (60 m × mm, ID) coated with 0.2% Ucon 50-HB-2000, a polypropylene glycol with 0.015% benzyltriphenylphosphonium chloride to aid in surface deactivation. The column

TABLE 2. NUMBER OF ANIMALS USED TO OBTAIN SAMPLES DISCUSSED IN TEXT

| | Intact | Castrate | Ovx[a] | Pnx | Anosmic |
|---|---|---|---|---|---|
| ♂ left sac, | | | | | |
| Feb. 5 | 5 | 0 | – | 1 | 0 |
| Feb. 25 | 3 | 1 | – | 0 | 2 |
| ♀ left sac, | | | | | |
| Feb. 5 | 2 | – | 1 | 1 | 0 |
| Feb. 25 | 1 | – | 1 | 1 | 1 |
| ♂ right sac, | | | | | |
| Feb. 10 | 4 | 1 | – | 0 | 1 |
| Feb. 18 | 5 | 0 | – | 1 | 0 |
| ♀ right sac, | | | | | |
| Feb. 10 | 2 | – | 0 | 2 | 1 |
| Feb. 18 | 4 | – | 1 | 1 | – |
| ♂ right sac, Aug. 4 | 3 | 2 | – | 2 | 0 |
| ♂ left sac, Aug. 20 | 3 | 2 | – | 2 | 0 |
| ♀ right sac, Aug. 4 | 4 | – | 1 | 0 | 0 |
| ♀ left sac, Aug. 20 | 2 | – | 1 | 1 | 0 |

[a]Ovx = ovariectomized; Pnx = pinealectomized.

was programed from 30 to 160°C at 2°/min. Compound identifications were carried out with nitrogen-selective (thermionic) and sulfur-sensitive (flame-photometric) detectors and through gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard 5982A dodecapole instrument. Chromatographic data (retention times and peak areas) were determined with a Perkin-Elmer Sigma 10 chromatography data station.

*Methodology for Acids.* We chose to convert the carboxylic acids to the benzyl esters in order to yield a less polar, less volatile derivative. The formation of benzyl esters was accomplished by a modification of the method of Liardon and Kihn (1978). An aliquot of sample (0.5 ml standard or anal gland suspension) was placed into a small, chromic acid-washed and silylated vial with a Teflon-lined screw cap. The solution was made basic (pH > 11) by the addition of one drop of 10 N NaOH to the vial and treated with two 1-ml aliquots of diethyl ether to remove any extractable neutrals or bases. The solution was then brought to pH 3 with aqueous HCl, and phenyldiazomethane (PDM) in ether was added until persistence of its orange-red color after a short and vigorous shaking. The vial was then placed into a water bath at 40°C and the contents allowed to react for 3 h with continuous stirring. During the reaction, more

PDM was added if the color had vanished. As the benzyl esters formed, they were extracted into the ether layer.

After 3 h, the aqueous layer was removed, the ether layer dried over anhydrous magnesium sulfate and transferred to a reacti-vial, and the volume adjusted to 0.4 ml. This solution was then chromatographed on a 27 m × 0.25 mm, ID, SE-30 glass capillary column in a modified Perkin-Elmer 3920 GC. A 0.2-$\mu$l sample was injected at room temperature, the oven brought to 60° at the appearance of the solvent peak, and the program at 2°C/min to 250°C begun. Final temperature was held for about $\frac{1}{2}$ hr. The complexity of the resulting chromatograms was derived to a large extent from the side reactions of the benzylating reagent, but the chromatographic resolution was good enough to permit detection of all the standard acid derivative peaks in the presence of those from the reagent and by-products. The same samples used for the anal-sac volatiles were examined for acids so that an analogous comparison could be accomplished. Peak areas and GC-MS data were obtained as discussed above. The identities of all acids found in the sample were verified by retention time comparison with standard acid derivatives.

*Antibiotic Treatments.* This experiment consisted of injecting 0.2 ml of an antibiotic suspension (tetracycline, 5 mg, and ampicillin, 10 mg) into one anal sac and 0.2 ml saline (to serve as control) into the other anal sac of two males and two females. Both sacs were emptied via the usual procedure before injection. The contents of the anal sacs were withdrawn 1 week after injection of the antibiotic, and the volatile components analyzed as described earlier. The treated and control anal-sac-sample chromatographic profiles were visually compared for each animal.

*Data Treatment.* For comparison of groups, the areas of 56 peaks were tabulated for each volatile profile. An average area and a variance for any given peak within a group (e.g., intact male in February) were calculated. A peak-for-peak comparison between groups was accomplished by computer. In each case, the $t$ test was performed to determine the significance at the 95% confidence level.

The left–right anal-sac designation was initially ignored so that larger data sets could be used in comparing male and female, etc., and to allow the use of the $t$ test for the August samples. After the group comparisons were made, the intact February groups (four samples) were separated into left (two samples) and right (two samples) categories (see Table 2) and the $t$ test performed as described above. Any left-vs.-right differences would then modify the results from the case where left and right were not designated.

## RESULTS AND DISCUSSION

*Volatiles.* A typical chromatogram of anal-gland volatiles is shown in Figure 1. Mass spectral identifications of numbered peaks are given in Table 3. One

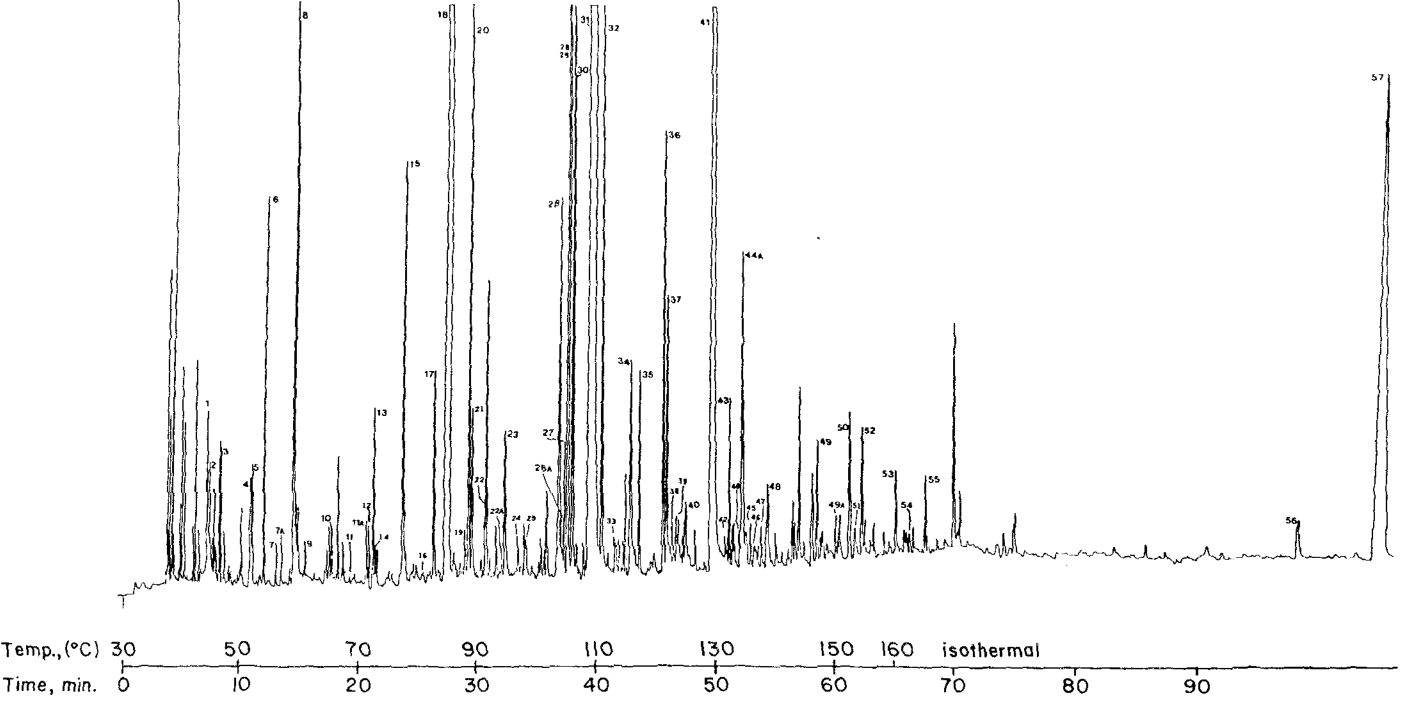


FIG. 1. Typical GC profile resulting from the separation of the volatile components of wolf anal-sac secretion.

TABLE 3. MASS SPECTRAL IDENTIFICATION OF PEAKS OF FIGURE 1

| | | | |
|---|---|---|---|
| 1 | Ethanol | 26A | 2-Nonanone |
| 5 | 4-Ethylcyclohexene | 27 | Acetate of unsaturated alcohol, e.g., 1-octen-3-ol |
| 6 | Toluene | | |
| 7 | An alkane | 28,29 | Isomers of 2-octenal |
| 7A | Propyl propionate | 30 | Benzaldehyde |
| 8 | Hexanal | 31 | 1-Octen-3-ol |
| 11A | Propyl 2-methylbutyrate | 32 | 1,4-Octadien-3-ol (proposed) |
| 12 | 2-Heptanone | 35 | 1-Octen-3-ol isobutyrate |
| 13 | Butyl butyrate | 36 | 2-Nonenal |
| 14 | Propyl pentanoate | 37 | 2-Octan-1-ol acetate |
| 15 | Ethyl hexanoate | 40 | 1-Octanol |
| 17 | *n*-Pentylfuran | 41 | 2-Octen-1-ol |
| 18 | 3-Octanone | 43 | 2-Octen-1-ol propionate |
| 19 | 2-Octanone | 44 | 2-Decenal |
| 20 | 1-Octen-3-one | 44A | 2-Octen-1-ol isobutyrate |
| 21 | An alkane | 45 | A dienol |
| 22 | Propyl isohexanoate | 49A | 2-Undecenal |
| 22A | Propyl hexanoate | 50 | Phenol |
| 23 | Butyl pentanoate | 52 | A dienal |
| 25 | Isobutyl 2-methylhexanoate | 56 | Indole |
| 26 | 3-Octanol | 57 | Diethyl phthalate (artifact) |

striking feature of the tabulation is the high percentage of compounds that are alcohols, aldehydes, or ketones, generally considered as "odoriferous compounds." Some of these compounds are closely related: for example, the appearance of 3-octanone, 1-octen-3-one, 1-octen-3-ol, 2-octenal, and 2-octen-1-ol, suggests that such compounds may be converted to a more reduced state (e.g., 2-octenal to 2-octen-1-ol) in the presence of anaerobic microflora (Scheline, 1973). This aspect will be discussed later.

Results of the group comparisons are summarized in Table 4. To condense our original data, peaks with very small areas (i.e., < 0.1–0.2 arbitrary units) were in many cases ignored due to the error associated with the integration of very small peaks which would leave a comparison suspect. It should be stated that such small peaks could be quite important to the overall scent, however. Table 4B contains entries (indicated by italics) that are not percentages, but rather areas (in arbitrary units). This was done because the peak concerned was not seen in the normal female, hence a percentage could not be calculated.

Using the intact male in February (♂ Feb., Table 4A) as a "standard," some interesting differences are observed: peak 12 is increased both in ♀ February and ♂̸ February, while peak 32 (tentatively, 1,4-octadien-3-ol) is not seen in the intact female or castrate male at all. In addition, *n*-pentylfuran, benzaldehyde, and a dienal (peak 52) are all increased in the female. A number of

TABLE 4A. SIGNIFICANTLY DIFFERENT GC PEAKS IN FEMALE AND ALTERED MALES AS COMPARED TO NORMAL MALES IN FEBRUARY[a]

| | | Relative amounts | | | | |
|---|---|---|---|---|---|---|
| ♂ Feb. peak no. | Compound | ♀ Feb. | ♂̸ Feb. | A ♂ Feb. | PNX ♂ Feb. | ♂ Aug. |
| 8 | Hexanal | – | – | – | 16 | – |
| 12 | 2-Heptanone | 187 | 231 | – | – | – |
| 13 | Butyl butyrate | – | – | – | – | 248 |
| 17 | *n*-Pentylfuran | 231 | – | – | – | 422 |
| 18 | 3-Octanone | – | – | – | 0 | – |
| 22 | Propyl isohexanoate | – | – | – | 0 | – |
| 28,29 | 2-Octenal | – | – | – | – | 22 |
| 30 | Benzaldehyde | 560 | – | 9 | – | 1207 |
| 32 | 1,4-Octadien-3-ol | 0 | 0 | – | – | – |
| 39 | 3,7-Dimethyl-2-octenal | – | – | – | 11 | – |
| 41 | 2-Octen-1-ol | – | – | – | 0 | – |
| 46 | – | – | – | 5 | 0 | – |
| 52 | A dienal | 512 | – | – | 0 | – |

[a]Entries represent the percentage of normal males.

TABLE 4B. SIGNIFICANTLY DIFFERENT GC PEAKS IN MALE AND ALTERED FEMALES AS COMPARED TO INTACT FEMALES IN FEBRUARY[a]

| | | Relative amounts | | | | |
|---|---|---|---|---|---|---|
| ♀ Feb. peak no. | Compound | ♀̸ Feb. | ♂̸ Feb. | A ♀ Feb. | PNX ♀ Feb. | ♀ Aug. |
| 7 | An alkane | – | 0 | – | – | 7.9 |
| 11 | | – | 0 | – | – | 0 |
| 12 | 2-Heptanone | 53 | – | – | – | 59 |
| 13 | Butyl butyrate | – | – | – | – | 181 |
| 17 | *n*-Pentylfuran | 43 | – | – | – | – |
| 20 | 1-Octen-3-one | – | – | – | 363 | – |
| 26 | 3-Octanol | – | *0.1* | *3.88* | *3.83* | – |
| 27 | 1-Octene-3-ol acetate | – | *0.49* | *0.76* | *0.18* | – |
| 28,29 | 2-Octenal | – | – | – | – | 567 |
| 30 | Benzaldehyde | 18 | 29 | 10 | 115 | 13 |
| 32 | 1,4-Octadien-3-ol | *0.88* | *0.98* | *0.1* | *2.49* | *0.44* |
| 36 | 2-Nonenal | – | – | – | 201 | – |
| 39 | 3,7-Dimethyl-2-octenal | – | 20 | – | – | – |
| 44 | 2-Decenal | *0.22* | *0.40* | *0.26* | *0.43* | – |
| 45 | A dienol | – | 43 | – | – | 29 |
| 52 | A dienal | 19 | – | – | – | – |

[a]Entries represent the percentage of normal females. Entries in italics are average peak areas (arbitrary units) because the indicated peak was not seen in intact females.

TABLE 4C. SIGNIFICANTLY DIFFERENT GC PEAKS IN FEMALES AND ALTERED MALES IN AUGUST COMPARED TO INTACT MALES IN AUGUST

| | | Relative amounts | | |
|---|---|---|---|---|
| ♂ Aug. peak no. | Compound | ♀ Aug. | ♂̸ Aug. | PNX ♂ Aug. |
| 7 | An alkane | – | – | 0 |
| 15 | Ethyl hexanoate | – | 49 | 13 |
| 17 | *n*-Pentylfuran | – | – | 9 |
| 28,29 | 2-Octenal | 1595 | – | – |
| 30 | Benzaldehyde | 6 | 6 | 19 |
| 36 | 2-Nonenal | 49 | – | 40 |
| 37 | 2-Octen-1-ol acetate | – | – | 0 |
| 45 | A dienol | 23 | – | 163 |

peaks were significantly different in the pinealectomized male in February. It is important at this point to stress that these numbers merely indicate trends; absolute quantitation was not intended nor should it be inferred due to the small sample size.

Comparing data to the normal female in February (Table 4B), we again see differences between the groups. Of most interest are peaks 30 (benzaldehyde), 32 (supposedly, 1,4-octadien-3-ol) and 44 (2-decenal). The first substance is diminished in the intact male and ovariectomized female, while the latter two are absent in the intact female. Peak 26 (3-octanol) and proposed 1-octen-3-ol acetate (peak 27) are both absent in the normal female but present in all the altered females to different degrees.

In the pinealectomized females, the significant volatiles were higher than in intact females (Table 4B). These observations are interesting in light of the fact that the pineal gland helps regulate reproduction (Reiter, 1980).

In February, benzaldehyde is much more abundant in the female than in

TABLE 4D. SIGNIFICANTLY DIFFERENT GC PEAKS IN ALTERED FEMALES IN AUGUST COMPARED TO INTACT FEMALES IN AUGUST

| | | Relative amounts | |
|---|---|---|---|
| ♀ Aug. | Compound | ♀̸ Aug. | PNX ♀ Aug. |
| 8 | Hexanal | 53 | – |
| 18 | 3-Octanone | 0 | – |

the male (Table 4A), while in August, the situation is dramatically reversed (Table 4C). Benzaldehyde is also significantly reduced in the castrate male in August compared to the intact male (Tables 4C and 4D). Finally, 2-octenal (peak 28) in August is higher in the intact female than in the intact male.

The differing levels of various compounds give reason to suspect that wolves use anal-sac secretions to convey messages. It is also plausible that subtle variation in the amounts of compounds present in the secretion could play a role in individual recognition. Because of the small number of samples, the conclusions here should be considered preliminary and interpreted with caution.

The results of the left–right anal sac comparison indicate some differences for intact animals in February (Table 5). These could be due partly to pH differences between glands (C. Asa, unpublished observation). However, this should not affect the conclusions derived from Table 4 (where left–right differences were ignored) because only four of these entries are left–right dependent. In addition, all of the affected entries of Table 4 are derived from at least one left and one right anal-gland sample so that unless a difference presented in Table 3 is slight, it is probably still significant.

If a chemical compound communicates a message, it is of interest to determine the biochemical origin of such a compound. The origins of many of the compounds seen in the volatiles are not clear; however, the pathways leading to the formation of some of the compounds have been described or hypothesized. It is thought that many of the ketone metabolites (3-octanone, 4-nonanone) arise from the decarboxylation of keto acids during fat metabolism in a manner analogous to the formation of acetone from acetoacetate (Liebich and Al-Babbili, 1975). This hypothesis is supported by an acute starvation study in rats, showing elevated ketone metabolites in urine during the first two or three days of starvation, when a rat is dependent on the oxidation of stored fat for energy metabolism (Parilla, 1978). Similarly, it is hypothesized that many of the aldehydes may be produced via decarboxylation of $\alpha$-keto acids derived from fatty acids

TABLE 5. SIGNIFICANT DIFFERENCES IN VOLATILE COMPONENTS OF LEFT AND RIGHT ANAL-GLAND SAMPLES FOR INTACT MALES AND FEMALES IN FEBRUARY

| Peak | Compound | ♂L | ♂R | ♀L | ♀R |
|---|---|---|---|---|---|
| 4 | | Absent | – | – | – |
| 7 | An alkane | – | 900% of L | – | 44% of L |
| 25 | Isobutyl 2-methylhexanoate | – | – | – | Absent |
| 28,29 | 2-Octenal | – | – | – | Absent |
| 39 | 3,7-Dimethyl-2-octenal | – | – | – | 31% of L |
| 40 | 1-Octanol | – | – | – | 530% of L |

or protein catabolism and amino acid transamination. It is also suggested that some aldehydes may arise from lipid peroxidation (Gurr and James, 1980).

*Volatiles Associated with Bacterial Action.* The hypothesis that some of these volatile components could be the result of bacterial action was mentioned earlier. It is known that phenol can arise from the microbial metabolism of tyrosine (Scheline, 1973), indole can result from fermentation involving tryptophan (Gunsalus and Stonier, 1961), and benzaldehyde can arise from numerous compounds including benzoic acid, benzyl alcohol, and mandelic acid (Scheline, 1973). It does not seem unreasonable that other compounds present could also be products of microbial metabolism. There is good reason for examining this aspect of the volatiles because many workers have hypothesized or shown the presence of bacteria in the anal sacs of numerous animals (Albone et al., 1974; Preti et al., 1976; Gorman et al., 1974; Albone and Perry, 1975).

Our data also indicate that some of the volatiles (especially 1-octen-3-one, 2-octenal, 2-octen-1-ol, and indole) are produced by microbes, since many of the listed peaks decreased or disappeared after antibiotic treatment (Table 6). We did find considerable individual variation; this was quite surprising, because one would expect a homogeneous bacterial population among conspecifics. (These data including the variability are supported by an earlier, unpublished version of the experiment.) However, a conclusion that could be drawn from our data is that the bacterial populations among wolves are not uniform. Based on the left–right comparisons made earlier, the effect due to these left–right differences on the present antibiotic data should be minimal. We are left, then, to deal with individual animal differences which could account for some of the

TABLE 6. QUALITATIVE CHANGES OBSERVED IN VOLATILE COMPONENTS OF NORMAL MALES, 213 R (LEFT CONTROL) AND 209 L (RIGHT CONTROL), NORMAL FEMALES, 212 R (LEFT CONTROL) AND 210 L (RIGHT CONTROL), VERSUS SAME ANIMAL CONTROL AS A RESULT OF ANTIBIOTIC TREATMENT OF ANAL SAC

| Compound | ♂ 213 R | ♂ 209 L | ♀ 212 R | ♀ 210 L |
|---|---|---|---|---|
| Dimethyl disulfide | eliminated | not seen | not seen | not seen |
| 1-Octen-3-one | decreased | present but unchanged | decreased | decreased |
| Dimethyl trisulfide | eliminated | not seen | not seen | not seen |
| 2-Octenal | decreased | present but unchanged | decreased | decreased |
| 1-Octen-3-ol | present but unchanged | present but unchanged | decreased | decreased |
| 2-Nonenal | decreased | present but unchanged | decreased | decreased |
| 2-Octen-1-ol | decreased | increased | decreased | decreased |
| Indole | eliminated | eliminated | eliminated | eliminated |

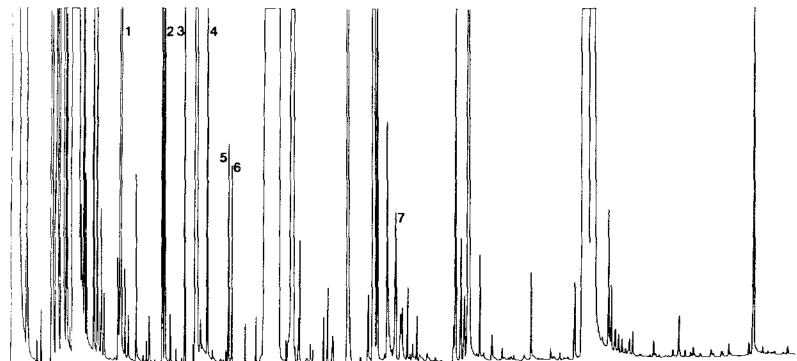


FIG. 2. Chromatogram resulting from the separation of the benzyl esters of acids present in anal gland secretion. Numbered peaks represent the benzyl esters of: 1, acetic acid; 2, propanoic acid; 3, 2-methylpropanoic acid; 4, butanoic acid; 5, 3-methylbutanoic acid; 6, 2-methylbutanoic acid; 7, benzoic acid.

variability in the compounds eliminated by the antibiotic treatment. This variability lends support to the individual recognition hypothesis.

Sulfur-containing compounds were not commonly seen in the volatiles of wolf anal-sac secretions but did appear occasionally. One such case is that of a male (Table 6) in which two such compounds were seen. Based on mass spectra, the first sulfur compound was identified as dimethyl disulfide and the second dimethyl trisulfide or 2,3,4-trithiapentane. Disulfides have been seen at low level in wolf urine volatiles (Raymer et al., 1981), but not the trisulfide. The disappearance of the compounds in the antibiotic-treated sample suggests a bacterial origin.

*Acids.* Acids found in the wolf anal-sac secretion were acetic, propanoic, 2-methylpropanoic, butanoic, 3-methylbutanoic, 2-methylbutanoic, and benzoic acids (Figure 2). Benzoic acid and the substituted butanoic acids were not observed by Peters (1974) in an earlier wolf gland study. On the other hand, we found no evidence of pentanoic acid, while Peters found pentanoic acid in one of the wolves he studied. It is obvious from Table 5 that the acids found in the present study are not unique and, furthermore, are probably produced (for the most part) from amino acids and bacteria (Albone et al., 1974., Preti et al., 1976; Gunsalus and Stonier, 1961; Gorman et al., 1974; Soketch, 1969).

In each acid profile from the wolf anal-sac samples, acetic acid yielded by far the most intense peak. This is not unexpected, since acetate is a common product of microbial fermentations. If we assume that the concentration of acetate at any one time is fairly stable due to its large abundance, all of the runs can be normalized by taking the area ratio of each acid in relation to acetate. By placing these runs on a similar scale, any significant patterns such as those

TABLE 7. SIGNIFICANT DIFFERENCES IN ACID LEVELS OF ANAL-SAC SECRETION AS DETERMINED BY COMPUTER COMPARISON OF VARIOUS CLASSES OF WOLVES

| Acid | Difference |
|---|---|
| Propanoic | PNX ♂ Aug. 199% of ♂ Aug. |
| 2-Methylpropanoic | ♀ Aug. 255% of ♀ Feb. |
| Butanoic | ♀ Aug. 220% of ♀ Feb. |
| 2-Methylbutanoic | PNX ♂ Feb. 234% of ♂ Feb. |
| 2-Methylbutanoic | ♀̸ Aug. 213% of ♀̸ Feb. |

discerned for the anal gland volatiles should become apparent after computer comparison as described in the experimental section.

The results (Table 7) show that significant variations in acid levels are fewer than seen for the other volatiles. Any acid level seen as greater in August than February can be explained partly by the fact that wolves expel anal-sac secretions less frequently in summer (C. Asa, unpublished observation). Thus secretions present in the anal sac may persist longer in summer, and ferment longer, yielding higher acid levels. This effect is not due to the internal temperature of the anal sac being higher in summer compared to winter as these temperatures are the same (C. Asa, unpublished observation).

The data do not indicate any significant differences in the acid levels between male, female, castrate male, and ovariectomized female, suggesting that anal-sac secretion acids may not help communicate sex or endocrine status in the wolves studied. The data are consistent with the suggestion by Gorman et al. (1974) that acids may play a role in individual recognition.

Left–right anal-gland comparisons were also made with acids using pooled samples from intact animals in February (Table 2). The only significant difference was in the level of benzoic acid. In females, benzoic acid from the right anal sac was 182% of that in the left, while in males, the level in the right anal sac was 30% of the level seen in the left. These differences, although statistically significant, are not particularly large, so no generalizations will be made.

## CONCLUSIONS

The volatile components of wolf anal-gland secretions were found to contain a large percentage of alcohols, aldehydes, and ketones. The compositions of the volatile components were significantly different between some groups, indicating that there may be a chemical basis for the use of the secretion for communication. Some of the volatile components apparently were produced by

bacteria. The acid components of the same samples were basically similar among the various groups, suggesting that acids present in wolf anal-gland secretion may not play a role in communicating sex or endocrine status, although they may function in individual identification.

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# INFLUENCE OF *Amaranthus hybridus* L. ALLELOCHEMICS ON OVIPOSITION BEHAVIOR OF *Spodoptera exigua* AND *S. eridania* (LEPIDOPTERA: NOCTUIDAE)[1]

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**Abstract**—Common pigweed, *Amaranthus hybridus* L., is a favorite host of the beet armyworm (BAW), *Spodoptera exigua* L. Chemicals extracted from homogenized pigweed with distilled water, ethanol, or dichloromethane and sprayed back on pigweed deterred oviposition by the BAW. Similarly, water extracts of frass from conspecific larvae or southern armyworm (SAW) larvae, *S. eridania* (Cramer), fed pigweed leaves and sprayed back on pigweed plants also deterred BAW oviposition, thus confirming that deterrence was due to plant allelochemics rather than specific compounds associated with the metabolic or excretory products of the larvae. Confirmation of the presence of oviposition-deterring chemicals in pigweed was used to explain a previously observed seasonal displacement of BAW by SAW on pigweed in the field.

**Key Words**—*Amaranthus hybridus, Spodoptera eridania, Spodoptera exigua*, Lepidoptera, Noctuidae, pigweed, oviposition behavior, oviposition deterrents, allelochemics, beet armyworm, southern armyworm.

## INTRODUCTION

Phytophagous insects use a wide variety of chemical cues to effect population density regulation. These may involve antiaggregation pheromones that specifically exclude one or both sexes (Rudinsky et al., 1974; Furniss et al., 1974; Byers, 1983), oviposition-deterring pheromones (Prokopy, 1975; McNeil and Quiring, 1983; Zimmerman, 1980, 1982), dispersion pheromones (Shorey, 1976),

[1] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

territorial marking pheromones (Cammaerts et al., 1977) and oviposition markers (Oshima et al., 1973; Kozlowski et al., 1983).

Oviposition deterrence in response to freshly damaged plants or frass from insect larvae fed plant material is another form of chemically mediated behavior that regulates insect populations. Unlike pheromones and other insect-produced bioregulators, such deterrents appear to be secondary plant substances that inhibit oviposition on contact with the insects at the ovipositional sites (Renwick and Radke, 1980, 1981; Rothschild and Schoonhoven, 1977; Sharma et al., 1981). There also is some evidence that vapors of secondary plant substances may deter oviposition by causing females to avoid plants infested with larvae (Chiang et al., 1960; Schurr and Holdaway, 1970).

The fact that host plant chemicals play a role in the spacing of phytophagous insects suggest that such chemicals may be useful in insect pest management. However, there is little information on which plants, or chemical constituents, might effectively inhibit oviposition by economically important insects. The beet armyworm (BAW), *Spodoptera exigua* (Hübner), is a cosmopolitan species that attacks more than 50 wild and cultivated plants in 18 families throughout its range (Mitchell, 1979). In an earlier study (Tingle et al., 1978), common pigweed, *Amaranthus hybridus* L., plants infested heavily with BAW larvae were not reinfested by this species but rather were succeeded by a related species, the southern armyworm (SAW), *S. eridania* (Cramer). This rather dramatic shift in species composition on *A. hybridus* suggested that the presence of feeding BAW larvae somehow decreased the attractiveness of the pigweed to gravid BAW females. The present study presents evidence of an insect oviposition regulator for the BAW in common pigweed, *A. hybridus*. The interaction of extracts of frass from BAW and SAW larvae fed pigweed on oviposition by the SAW and BAW, respectively, also is reported.

## METHODS AND MATERIALS

Fresh leaves of *A. hybridus* (20 g) were homogenized in an electric blender with 100 ml of distilled water ($H_2O$), ethanol (EtOH), or dichloromethane ($CH_2Cl_2$) for 30 sec, then filtered through a white Viva® paper towel. The $H_2O$ extract was brought back to original volume with $H_2O$ before spraying onto the plants. The EtOH and $CH_2Cl_2$ extracts were evaporated to dryness in a rotoevaporator and redissolved in 100 ml of 3 : 1 acetone–$H_2O$ for spraying.

Frass was collected from large BAW larvae (i.e., late 3rd, 4th, and 5th instars) fed fresh leaves of nonflowering *A. hybridus* plants. The larvae were obtained from laboratory cultures reared on pinto bean artificial diet. The frass was collected daily and stored in a sealed jar in a freezer until used. Twenty grams of frass were homogenized in an electric blender with 100 ml of $H_2O$, EtOH, or $CH_2Cl_2$. The material was filtered and prepared for spraying as de-

scribed previously. Collection of larval frass from the SAW and its preparation for spraying was the same as described for the BAW.

Potted *A. hybridus* plants (ca. 40 cm high) were sprayed with 25 ml of the test material using a Badger® air brush (model 250). Both the upper and lower leaf surfaces were sprayed. Preliminary trials indicated that neither $H_2O$ nor the acetone–$H_2O$ mixture had any significant effect on oviposition; therefore, the controls were not sprayed. The treated plant and corresponding control plant were placed in opposite corners of a 61 × 61 × 61-cm screenwire cage (ca. 34 cm between plants) containing 20–30 pairs of 2- to 3-day-old BAW or SAW moths reared in the laboratory on pinto bean artificial diet. Fresh insects were added daily to keep the moth population at the desired level. All tests were carried out in the greenhouse under natural light at ca. 23°C at night and 27°C during the day. Each test was replicated 8–15 times. The cages were set over greenhouse benches lined with plastic and filled with water to keep the relative humidity high ($>80\%$).

The response of gravid BAWs to *A. hybridus* plants treated with different dosages of ethanol extracts from homogenized *A. hybridus* leaves were carried out using the procedures cited above. Five different dosage levels were tested—2.5, 5, 10, 20, and 40 g/100 ml ethanol—and each dosage was replicated 8–10 times.

Two experiments were conducted in an attempt to define the behavioral response of BAW females to the deterrent chemicals found in *A. hybridus* leaf extracts. First, an ethanolic extract of fresh pigweed leaves (20 g/100 ml) prepared as described previously was evaporated from an aluminum gelatin mold (7.5 cm diam, 4 cm deep) glued to a wooden stake. The mold was positioned adjacent to the stalk just below the bottom leaf of a pigweed plant. The test plants were stripped to six leaves approximately equal in size before use. Twenty-five milliliters of the extract was placed in the treatment mold, and an equal quantity of ethanol was placed in the mold beneath the control plant. One treated and one control plant were placed in opposite corners of the test cages (15 replicates), each containing 20–30 pairs of 2- to 3-day-old BAW moths. The total number of egg masses on the treated and control plants in each cage was recorded.

In the second experiment, the test plants were stripped to six leaves as before, and three leaves on each plant were sprayed with leaf extract. Each of the three control leaves on a plant were enclosed in plastic bags during spraying. The three treated leaves on each plant received a total of ca. 12.5 ml of spray. Two similarly treated plants were placed in opposite corners of each cage. Thus, the two plants received a total of 25 ml of spray, which was the same quantity used in the evaporation experiment and also the quantity sprayed on a single plant in the test of different solvent systems. The number of egg masses on the treated and control leaves on each plant was recorded; each plant was considered a separate replicate (2 plants/cage, total 10 replicates).

The plants were sprayed at ca. 1500 hr and placed immediately into the test cages. The plants were examined the following morning, and the number of egg masses on each plant was counted after which the plants were discarded. Replicates having fewer than a total of 20 egg masses were discarded. For statistical analysis, the data were converted to percentages of the total number of egg masses deposited on the treated and control plants in each replicate. Differences between paired means were separated using Student's $t$ test.

## RESULTS

The percentage of egg masses deposited by the BAW on pigweed plants treated with leaf extracts or frass was reduced significantly compared to the controls in each of the three solvent systems evaluated (Table 1). Ethanol extracts gave the greatest reductions, although the differences between solvent systems were minor. Extracts of pigweed leaves were tested at five dosage levels: 2.5, 5, 10, 20, and 40 g/100 ml ethanol. Only the 2.5-g level failed to deter oviposition by the BAW, and there was no significant difference in the level of deterrence recorded at the 5- to 40-g dosages (Figure 1).

An $H_2O$ extract of frass from SAW larvae fed pigweed leaves also deterred oviposition by the BAW. In this test, BAW moths deposited 65.8% of the total

TABLE 1. EFFECT OF EXTRACTS FROM HOMOGENIZED FRESH *Amaranthus hybridus* LEAVES AND FRASS FROM BEET ARMYWORM LARVAE FED *A. hybridus* LEAVES ON OVIPOSITION BY BEET ARMYWORM

| Solvent | Total no. egg masses | % Total egg masses (± SE)[a] | | Percentage reduction[b] |
|---|---|---|---|---|
| | | Treatment | Control | |
| Leaf extract[c] | | | | |
| Distilled water | 923 | 30.7 ± 5.6 | 69.3 ± 5.6** | 55.7 |
| Ethanol | 681 | 23.4 ± 3.8 | 76.6 ± 3.8** | 69.4 |
| Dichloromethane | 1,000 | 34.6 ± 5.2 | 65.4 ± 5.2** | 47.1 |
| Frass extract[c] | | | | |
| Distilled water | 1,203 | 30.0 ± 6.2 | 70.0 ± 6.2** | 57.1 |
| Ethanol | 678 | 17.4 ± 7.2 | 82.6 ± 7.2** | 78.9 |
| Dichloromethane | 554 | 27.7 ± 6.2 | 72.3 ± 6.2** | 61.7 |

[a] Means followed by ** differ significantly from control at the 1% level, Student's $t$ test.

[b] $$\% \text{ Reduction} = \frac{\% \text{ egg masses on control} - \% \text{ egg masses on treatment}}{\% \text{ egg masses on control}} \times 100.$$

[c] Twenty g/100 ml solvent.

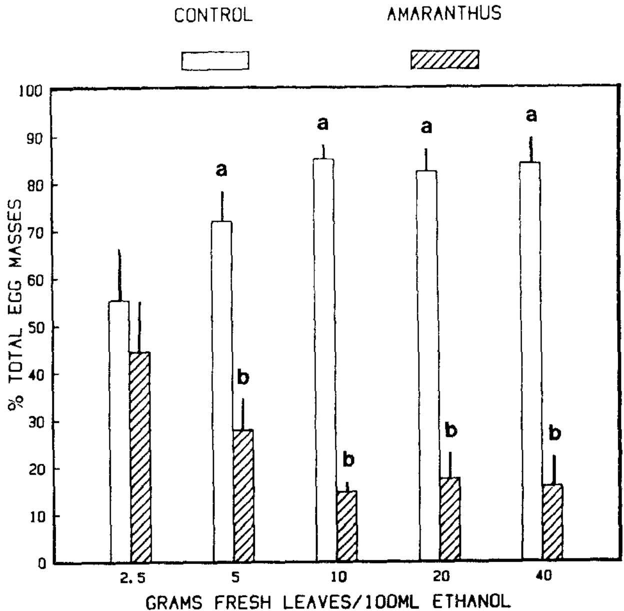


FIG 1. Response of gravid beet armyworms to *Amaranthus hybridus* plants treated with different dosages of ethanol extracts from homogenized *A. hybridus* leaves. Each plant was treated with 25 ml of extract. Means within dosage levels followed by different letters differ significantly at the 1% level, Student's $t$ test. Narrow bars indicate standard errors of the means. The total number of egg masses recorded in tests at each dosage level was: 2.5 g, 533; 5 g, 836; 10 g, 266; 20 g, 562; 40 g, 273.

number of egg masses (740) on control plants and 34.2% on treated plants ($t$ = 2.466, $p < 0.05$, 13 replicates). The 48% reduction in oviposition by BAW on SAW frass-treated plants in this test was about two thirds of that recorded for an $H_2O$ extract of frass from conspecific larvae (Table 1). Similarly, an $H_2O$ extract of frass from BAW larvae fed pigweed leaves sprayed on *A. hybridus* plants deterred oviposition by the SAW. Of the total number of egg masses (255) deposited by the SAW, 75.8% were on the control plants and 24.2% were on the treated plants ($t = 3.317$, $p < 0.01$), a reduction of 68.1%. The $H_2O$ extracts of BAW or SAW frass reduced BAW and SAW oviposition at levels similar to $H_2O$ extracts of homogenized *A. hybridus* leaves. These results confirm that oviposition deterrence in both species was due to plant allelochemics rather than specific chemicals associated with the metabolic or excretory products of feeding larvae.

There was no significant difference in the percentage of egg masses deposited by BAWs on pigweed plants where the ethanol extract of pigweed leaves was evaporated from gelatin molds placed beneath the plants. A total of 1130

egg masses was recorded, of which 54% were deposited on the treated plants and 46% were deposited on the corresponding controls. When alternate leaves of the pigweed plants were sprayed with an ethanol extract of pigweed leaves, BAW moths deposited 99% of the total number of egg masses recorded (372) on the adjacent untreated leaves ($t$ = 51.466 $p < 0.01$, 10 replicates). The distance between treated and untreated leaves on the same plant rarely exceeded 2.5 cm. These results suggest that deterrence was due to contact chemoreception rather than olfactory chemoreception. However, no other spacing between leaves was evaluated. Therefore, it is possible that the moths responded to a gradient of deterrent where the control leaves were more acceptable because of the lower concentration of volatiles reaching them.

## DISCUSSION

The term "allelochemic" is used to describe chemicals that mediate interspecific interactions and is defined as a chemical that is significant to organisms of a species for reasons other than food (Whittaker, 1970). Many plants contain secondary substances that deter oviposition in phytophagous insects (Gupta and Thorsteinson, 1960; Yamamoto and Fraenkel, 1960; Hsiao and Fraenkel, 1968). These natural chemicals may be present in suitable (Renwick and Radke, 1981) or unsuitable host plants (Lundgren, 1975; Tingle and Mitchell, 1984). With the BAW and the SAW, the deterrent substances from pigweed appear to be unaltered plant compounds and not metabolic by-products of conspecific larvae fed *A. hybridus* leaves. It is important to note that the deterrent compounds apply only to damaged, i.e., homogenized or digested, plant material, indicating that the active chemicals probably are not cuticular componets. It is obvious that if these chemicals were on the leaf surface they would have deterred oviposition on undamaged plants.

The deterrent substances were found both in plants collected from the field and plants grown from seed in a greenhouse, suggesting that such chemicals are a part of the "normal" chemical profile of *A. hybridus* and not defensive substances that were produced when the plants were under stress of attack, as suggested by Maugh (1982). However, we did not determine the chemical profile for this species.

*Amaranthus hybridus* occurs primarily in disturbed soils such as cultivated fields and along fence rows (Fernald, 1950). During late spring and early summer in north-central Florida, *A. hybridus* often grows to heights of 2 m or more. As the season progresses and day length becomes shorter, *A. hybridus* begins to flower. New plants that emerge in late summer and fall are much shorter and flowering starts at a much earlier age.

In north-central Florida, *A. hybridus* has three principal pests, namely, the BAW, SAW, and the southern beet webworm (SBWW), *Herpetogramma bi-*

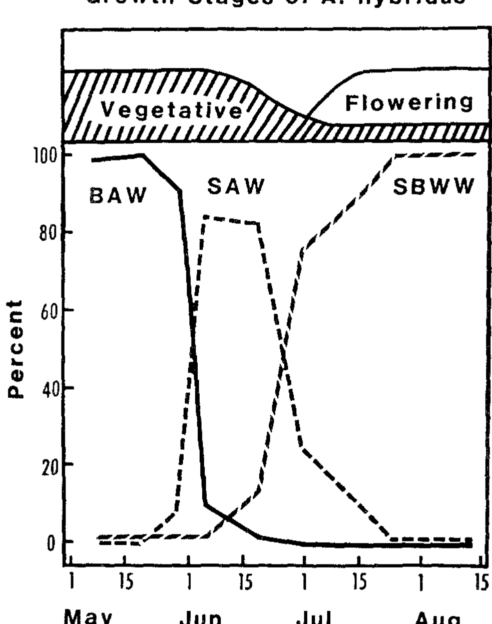


FIG. 2. Seasonal occurrence of beet armyworm (BAW), southern armyworm (SAW), and southern beet webworm (SBWW) on common pigweed, *Amaranthus hybridus*, in north-central Florida as related to plant growth stages (after Tingle et al., 1978).

*punctalis* (F.) (Tingle et al. 1978). The BAW is predominant in May, the SAW in June, and the SBWW is predominant in July and August (Figure 2). The following is offered as a possible explanation for the displacement of the BAW by the SAW on *A. hybridus*.

The succession of insects, i.e., BAW and SAW, that attack *A. hybridus* may be regulated by the substances that deter oviposition. Both the BAW and SAW are migrants that overwinter only in tropical or subtropical environments (Mitchell 1979). The BAW generally moves into north-central Florida earlier in spring than the SAW (unpublished data) and oviposits on *A. hybridus*. Larval populations per plant often are very high during this period, resulting in severe defoliation. Moths that emerge early from pupae developed from this initial colonization most probably are deterred from ovipositing on *A. hybridus* by the exudates of the severely damaged leaves combined with large quantities of frass from mostly large, voraciously feeding BAW larvae. As the later developing BAW larvae leave the plants to pupate, the wounds on the leaves heal and the frass load on the plants declines due to the action of wind and moisture (dew, rain). Arriving SAW moths then oviposit on these plants, although there may be some BAW larvae present. Late emerging BAW moths presumably then are

deterred from ovipositing on *A. hybridus* due to the activity of the vigorously feeding SAW larvae that deposit large quantities of frass and because of the exudates of the wounded leaf tissue. As *A. hybridus* enters the reproductive state, the plants are colonized by yet a third species, the SBWW (Tingle et al., 1978). There usually is an abundance of *A. hybridus* in all stages of development, as well as other potential wild and cultivated host plants available to both the BAW and SAW during late summer and fall.

Although the displacement of BAW on *A. hybridus* by SAW possibly may be due to a fortuitous set of circumstances whereby the SAW arrives in the area about the time that the BAW is completing its development on *A. hybridus*, this alone would not explain why the plants are not reinfested by BAW. Therefore, it appears reasonable to assume that oviposition-deterring chemicals released by SAW larvae feeding on *A. hybridus* do indeed contribute significantly to the displacement of the BAW. The scenario described here also could explain why certain insect pests tend to leave heavily damaged monocultures and "migrate" to more acceptable hosts in the same or different areas as described for the European corn borer (ECB), *Ostrinia nubilalis* (Hübner), in corn (Schurr and Holdaway, 1970). It is not unreasonable, therefore, to assume that other insect pests also may behave in a similar manner, especially where host plants are under severe attack.

The plant kingdom is a vast warehouse of chemical substances manufactured and used by plants for defense from insect attack. Oviposition deterrence is but one of several defensive mechanisms. However, it is potentially a very important pest control device. Chemically defined allelochemics from plants that deter oviposition on contact may be used to control insect pests, provided such chemicals are nonhazardous to humans, are ecologically safe, and are inexpensive to produce.

Wild and cultivated plant hosts could serve as an important reservoir of deterrent compounds. Recent advances in molecular genetics and plant cloning techniques possibly could be used to increase the deterrent compounds and even turn some "weeds" into important cash crops from which the deterrent compounds could be extracted in quantity. This technique would be especially useful for active compounds whose chemical structures might be too complicated for large-scale synthesis (Klocke and Kubo, 1982).

Plant allelochemics such as the oviposition-deterring substances for the BAW and SAW obtained from *A. hybridus* could have additional benefits. For example, Altieri et al. (1981) showed that application of $H_2O$ extracts of *Amaranthus* sp. enhanced parasitization of *Heliothis zea* (Boddie) eggs by naturally occurring and artificially released *Trichogramma* sp. in a range of crops. The frass of larvae feeding on plants also is known to contain kairomones that elicit host-seeking behavior in parasitic wasps (Lewis it al., 1976). It remains to be determined, however, whether the chemicals that influence parasite behavior are the same as those responsible for modified oviposition behavior in pest insects.

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# EFFECTS OF VARIOUS MIXTURES OF FERULIC ACID AND SOME OF ITS MICROBIAL METABOLIC PRODUCTS ON CUCUMBER LEAF EXPANSION AND DRY MATTER IN NUTRIENT CULTURE[1]

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**Abstract**—Cucumber seedlings (*Cucumis sativus* cv. 'Early Green Cluster') ranging from 6 to 16 days of age were treated with various concentrations (0–1 mM) of caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, and vanillic acids and mixtures of ferulic acid and one or two of the other phenolic acids. Seedlings were grown in full-strength Hoagland's solution which was changed every other day. Phenolic acid treatments were given with each nutrient solution change starting at day 6 or given once when seedlings were 13 or 14 days old. Leaf area, mean relative rates of leaf expansion, transpiration rates, water utilization, and the concentrations of the phenolic acids in nutrient solution were determined at one- or two-day intervals. Seedling dry weight was determined at final harvest. Seedling leaf area and dry weight were linearly related. Since leaf areas can be easily obtained without destructive sampling and leaf area expansion responds rapidly to phenolic acid treatments, it was utilized as the primary indicator of plant response. The resulting data suggested that a number of ferulic acid microbial metabolic products, as well as two other phenolic acids observed in soils (*p*-coumaric and syringic acid), can reduce seedling dry weight, leaf expansion, and water utilization of cucumber seedlings in a similar manner. The magnitude of impact of each of the phenolic acids, however, varied with phenolic acid and concentration. It appears that the inhibitory activity of these phenolic acids involved water relations of cucumber seedlings, since the phenolic acid treatments resulted in closure of stomata which then remained closed for several days after treatment. The data also demonstrated that the effects of mix-

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tures of phenolic acids on cucumber seedlings may be synergistic, additive, or antagonistic. The type of response observed appeared to be related to the factor measured, the compounds in the mixture, and the magnitude of inhibition associated with each compound. The data also indicated that the effects of the various phenolic acids were reversible, since seedling leaf area increased rapidly once phenolic acids were removed from the root environment. Mean relative rates of leaf expansion recovered even in the presence of the various phenolic acids.

**Key Words**—Phenolic acids, cinnamic acid derivatives, benzoic acid derivatives, allelopathy, leaf area, mean relative rates of leaf area expansion, cucumber seedlings, phenolic acid mixtures.

## INTRODUCTION

Previously, we had shown that cucumber radicle length, absolute and relative rates of leaf expansion, leaf area, and plant dry weight were reduced by millimolar concentrations of ferulic acid (Blum et al., 1984; Blum and Dalton, 1985). We used cucumber as our bioassay species because of its sensitivity to ferulic acid, its rapid growth rate, and its predictible behavior. We chose to work with ferulic acid because this allelopathic compound (Wang et al., 1967; del Moral and Muller, 1970; McPherson et al., 1971; Rasmussen and Einhellig, 1977; Patterson, 1981) is commonly found in plants (Bates-Smith, 1956), has been isolated from soil (Whitehead, 1964; Guenzi and McCalla, 1966a,b; Wang et al., 1967; Lodhi, 1975; Whitehead et al., 1981 and 1982), and is a product of lignin degradation (Flaig, 1964; Turner and Rice, 1975; Martin and Haider, 1976).

Microbial metabolism of ferulic acid results in the formation of caffeic, protocatechuic, *p*-hydroxybenzoic, and vanillic acids (Evans, 1963; Flaig, 1964; Dagley, 1971; Turner and Rice, 1975; Martin and Haider, 1976; Blum et al., 1984; Blum and Dalton, 1984). In addition to these phenolic acids, two other compounds (i.e., *p*-coumaric and syringic acids) have also frequently been isolated from soils (Guenzi and McCalla, 1966a,b; Wang et al., 1967; Whitehead et al., 1981, 1982). All of these compounds have been implicated as allelopathic agents (Rice, 1984). Since plant roots encounter these substances in various combinations and concentrations in the soil environment, an understanding of how various mixtures of such phenolic compounds may affect plant growth and development is an essential step in understanding their potential role as allelopathic agents in soil environments. The effects of mixtures of phenolic acids on plant growth may be: (1) equal to the sum of the effects of each phenolic acid tested separately (i.e., additive), (2) lower than the sum of the effects of each phenolic acid tested separately (i.e., antagonistic), or (3) greater than the sum of the effects of each of the phenolic acids tested separately (i.e., synergistic). All three effects have been noted for mixtures of phenolic acids on growth (Rasmussen and Einhellig, 1977; Einhellig et al., 1982; Blum et al., 1984).

The specific objective of this research was to determine how various mixtures of phenolic acids might affect cucumber leaf expansion and dry matter production in nutrient culture. The phenolic acids chosen for this study were: caffeic, ferulic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic, sinapic, syringic, and vanillic acids. The long-term objective of this project was (Dalton et al., 1983; Blum et al., 1984; Blum and Dalton, 1985), and is, to develop a model system that may be used to gain a better understanding of the effects of allelopathic agents on plant growth and development in soil systems.

## METHODS AND MATERIALS

*General Aspects.* All cucumber seeds (*Cucumis sativis* cv. 'Early Green Cluster') were germinated in the dark at 28–30°C in trays containing sterile vermiculite and full-strength Hoagland's solution (Hoagland and Arnon, 1950). Cucumber seeds were obtained from Wyatt Quarles Seed Company, Raleigh, North Carolina. After 48 hr, the seedlings were transferred to 120-ml glass snap-cap bottles containing full-strength Hoagland's solution. Seedlings were suspended in nutrient solution by a foam collar through a hole in the cap of the bottles. The snap-cap bottles were placed under light banks in the laboratory that provided 150 $\mu$Einsteins/m$^2$/sec for 12 hr/day (Blum and Dalton, 1985). The temperature under the light banks ranged from 21°C to 30°C for all experiments. Seedlings were treated with phenolic acids dissolved in full-strength Hoagland's solution. All solutions were adjusted to a pH of 5.8. Solutions in the bottles were replaced every other day. Phenolic acids, except for sinapic acid, were obtained from Sigma Chemical Company, St. Louis, Missouri. Sinapic acid was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Each time solutions were changed, the length and width of each true leaf was measured. Dry weights of shoots and roots (dried at 50°C for 48 hr) were determined at harvest.

*Multiple Treatments.* Six-day-old seedlings (total number of plants = 75) were treated with nutrient solutions containing 0.0, 0.25, 0.5, and 1 mM caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, protocatehuic, sinapic, syringic, and vanillic acids. The level of the phenolic acids and their breakdown products was monitored for the 1 mM treatments prior to each solution change, using a Waters fully automated high-performance liquid chromatograph (HPLC). Separation of the various phenolic acids was achieved by binary gradient elutions through a Radial-Pak $\mu$-Bondapak $C_{18}$ (reverse-phase) cartridge with a flow of 2.5 ml/min. Solvent A consisted of a mixture of 2.5% methanol, 0.25% ethyl acetate and 0.5% acetic acid. Solvent B was a mixture of 80% methanol, 1% ethyl acetate and 2% acetic acid. Gradient elutions of the samples were achieved by maintaining solvent A for 20 min and then gradually increasing solvent B to 98%. The total run time was 80 min. Identification of the phenolic acids in the samples

was made by comparing retention times and the ratios of absorbance between 280 and 254 nm with those of known standards. Seedlings were harvested at day 18.

*Phenolic Acid Mixtures.* Thirteen-day-old seedlings (total number of plants = 72) were treated once for two days with 0.0, 0.5, and 1 mM solutions of the phenolic acids listed previously and mixtures (0.5 + 0.5 mM) of ferulic acid plus one other phenolic acid. Seedlings were returned to Hoagland's solution without phenolic acids after the two-day treatment. Seedlings were harvested when 19 days old.

In a second experiment 14-day-old seedlings (total number of plants = 81) were treated once with 0.0, 0.125, 0.25, 0.5, 0.75, and 1 mM of ferulic, *p*-coumaric, and vanillic acids; mixtures of ferulic-*p*-coumaric acid, ferulic–vanillic acid, and *p*-coumaric–vanillic acid; and a mixture of all three acids. Seedlings were returned to Hoagland's solution without phenolic acids after two days. Mixtures consisted of equivalent millimolar concentrations for each phenolic acid. The concentrations were as follows: two-way mixtures—0.125, 0.25, and 0.5 mM; the three-way mixtures—0.167 and 0.333 mM. The solution levels in each bottle were measured with a millimeter ruler prior to each solution change. Seedlings were harvested on day 18.

*Transpiration.* Fourteen-day-old-seedlings were treated once or twice two days apart with 0.5 or 1 mM ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids. Each treatment lasted two days. Water loss from the first true leaf was measured once a day between 9 and 10 AM for 9 days with a Li-Cor Steady State Porometer (model Li-1600) under one of the lightbanks (150 $\mu$Einsteins/$m^2$/sec). Since this was a preliminary experiment, plants for each treatment, except for the control plants, were not replicated (total number of plants = 24).

*Data Analysis.* Treatments for each experiment were randomly distributed under each of three light banks. Unless otherwise stated, all experimental treatments had an *N* value of 3. Data were analyzed using the Statistical Analysis System (SAS) programs for analysis of variance and linear and multiple regressions (Helwig and Council, 1979). Least significant differences ($LSD_{0.05}$), where presented, are provided only as a measure of experimental precision. Inferences are based on the appropriate analysis of variance or regression analysis.

Leaf area was determined from length and width measurements of leaves and the equation previously described by Blum and Dalton (1985). Mean relative rates of leaf expansion were defined by the equation:

$$\text{Mean relative rate} = \ln(\text{leaf area at time}_{x+1} + 1) - \ln(\text{leaf area at time}_{x} + 1)$$

Since all readings were taken at two-day intervals, values given were based on these two-day periods. One was added to each value because zero values were not uncommon for the early growth periods, and the logarithm of zero is undefined. For additional details about growth analysis see Radford (1967) or Evans (1972).

Volumes of the solutions in the snap-cap bottles were determined from solution levels in millimeters and the following equation:

$$\text{ml of solution} = 13.78 + 1.5\ (\text{level in mm}),\ \alpha = 0.0001,\ R^2 = 0.99,\ N = 36$$

## RESULTS AND DISCUSSIONS

*Multiple Phenolic Acid Treatments.* Multiple treatments of all eight phenolic acids reduced leaf area and plant dry weights (Figure 1, Table 1). *p*-Hy-

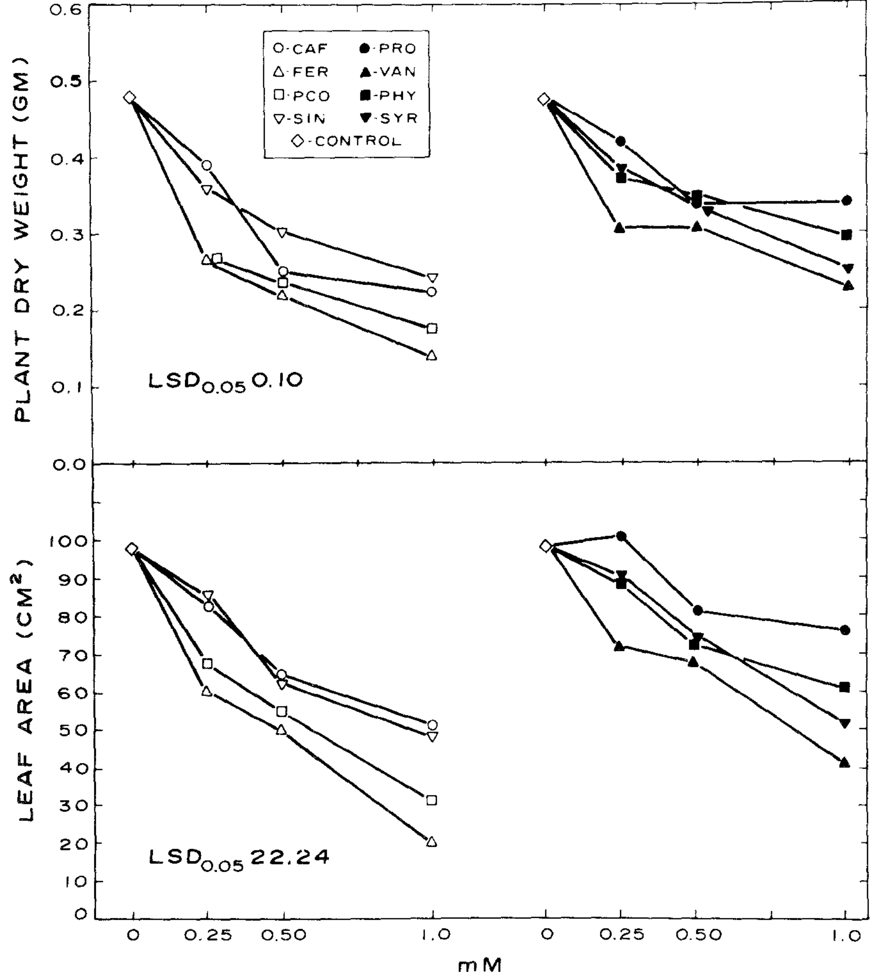


Fig. 1. Effects of multiple treatments of various concentrations of eight phenolic acids on dry weight and leaf area of 18-day-old cucumber seedlings. Treatments were initiated when seedlings were 6 days old ($N = 3$).

TABLE 1. PARTIAL REGRESSION COEFFICIENTS AND $R^2$ VALUES FOR LEAF AREA AND PLANT, SHOOT AND ROOT DRY WEIGHT OF CUCUMBER SEEDLINGS TREATED WITH VARIOUS PHENOLIC ACIDS (0, 0.25, 0.5 AND 1 mM)[a]

| Phenolic acids | Plant leaf area ($cm^2$) | | | | Plant dry weight (g) | | | |
|---|---|---|---|---|---|---|---|---|
| | Intercept | Linear | Quadratic | $R^2$ | Intercept | Linear | Quadratic | $R^2$ |
| CAF[b] | 94.72 | -46.81 | | 0.63 | 0.44 | -0.25 | | 0.55 |
| FER | 89.09 | -72.30 | | 0.80 | 0.46 | -0.72 | 0.41 | 0.85 |
| PCO | 90.35 | -62.28 | | 0.73 | 0.45 | -0.69 | 0.42 | 0.77 |
| PHY | 96.58 | -35.87 | | 0.40 | 0.44 | -0.17 | | 0.42 |
| PRO | 100.35 | -25.42 | | 0.38 | 0.44 | -0.12 | | 0.41 |
| SIN | 95.70 | -49.45 | | 0.74 | 0.44 | -0.22 | | 0.67 |
| SYR | 98.89 | -47.60 | | 0.65 | 0.45 | -0.21 | | 0.65 |
| VAN | 93.94 | -53.13 | | 0.81 | 0.42 | -0.20 | | 0.56 |
| | Shoot dry weight (g) | | | | Root dry weight (g) | | | |
| CAF | 0.37 | -0.23 | | 0.56 | | | | |
| FER | 0.39 | -0.65 | 0.37 | 0.85 | 0.06 | -0.04 | | 0.76 |
| PCO | 0.39 | -0.65 | 0.41 | 0.79 | 0.06 | -0.03 | | 0.63 |
| PHY | 0.38 | -0.15 | | 0.45 | | | | |
| PRO | 0.38 | -0.12 | | 0.46 | | | | |
| SIN | 0.38 | -0.21 | | 0.69 | | | | |
| SYR | 0.38 | -0.21 | | 0.70 | | | | |
| VAN | 0.36 | -0.19 | | 0.59 | | | | |

[a]Seedlings were initially treated when six days old. Solutions were changed every other day. Plants harvested when 18 days old.
[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SIR = syringic acid, VAN = vanillic acid.

droxybenzoic, protocatechuic, and syringic acids were the least inhibitory (17–29% reductions at 0.5 mM) and ferulic and *p*-coumaric acids were the most inhibitory (44–52% reduction at 0.5 mM). The effects of the two latter compounds were also distinct from the other six in that they inhibited root dry weight and that their impact on shoot and plant dry weight was best described by a quadratic instead of a linear equation.

The eight phenolic acids used in this study included both cinnamic and benzoic acid derivatives. Based on ring substitutions, the cinnamic and benzoic acid derivatives can be paired as follows: ferulic + vanillic, caffeic + protocatechuic, *p*-coumaric + *p*-hydroxybenzoic, and sinapic + syringic acids, respectively. Although it was not possible to identify the roles, if any, of the various substitutions on the benzene rings, the data do indicate that the cinnamic acid derivatives were more inhibitory to cucumber seedlings than the corresponding benzoic acid derivatives.

In a previous publication (Blum and Dalton, 1985), we noted a rapid decline of ferulic acid from nutrient solutions containing cucumber seedlings. This was also observed for all the phenolic acids used in this study. By the time plants reached 9–11 days of age, the phenolic acids supplied every other day were completely gone from solution after 48 hr with the exception of syringic and ferulic acid. Maximum reduction observed over a 48-hr period for syringic and ferulic acid was 65% and 88%, respectively. Cucumber seeds frequently have associated with them substantial microbial populations which are found both externally and internally (Leben, 1961; Mundt and Hickle, 1976). Since our solution cultures contained microorganisms associated with the cucumber seeds, it was not possible to separate the disappearance of the phenolic acids from the solutions due to microbial activity from that due to root adsorption and/or absorption. Microbial metabolic products were identified in the solutions of the 1 mM treatments. It is possible, that at least some of these "microbial" products may have been a product of root uptake, conversion, and exudation and/or synthesis by seedlings under phenolic acid-induced stress. No phenolic acids were detected in the Hoagland's solution of the control seedlings. The following compounds were identified in the various phenolic acid solutions: ferulic acid solution—protocatechuic and vanillic acid; *p*-coumaric acid solution—*p*-hydroxybenzoic acid; caffeic acid solution—cinnamic and protocatechuic acid; *p*-hydroxybenzoic acid solution—protocatechuic acid; and sinapic acid solution—cinnamic, ferulic, and syringic acid. Concentrations of the breakdown products ranged from a trace to 26 $\mu$g/ml. The microbial metabolic products, ferulic, *p*-hydroxybenzoic, protocatechuic, syringic, and vanillic acids, increased for a number of phenolic acid treatments with each solution change. Maximum concentrations observed for these compounds were 4, 26, 20, 12, and 11 $\mu$g/ml, respectively.

Mean relative rates of leaf expansion ($cm^2/cm^2/2$ days) were calculated to determine the effects of the various phenolic acids on leaf expansion and to determine if such growth effects were maintained with each subsequent solution change. Mean relative rates of leaf expansion were significantly inhibited by five of the eight phenolic acids upon initial treatment (Figure 2, Table 2). However, this inhibition was rapidly lost (note positive partial regression coefficients) in spite of the continued treatments of seedlings with fresh solutions of the phenolic

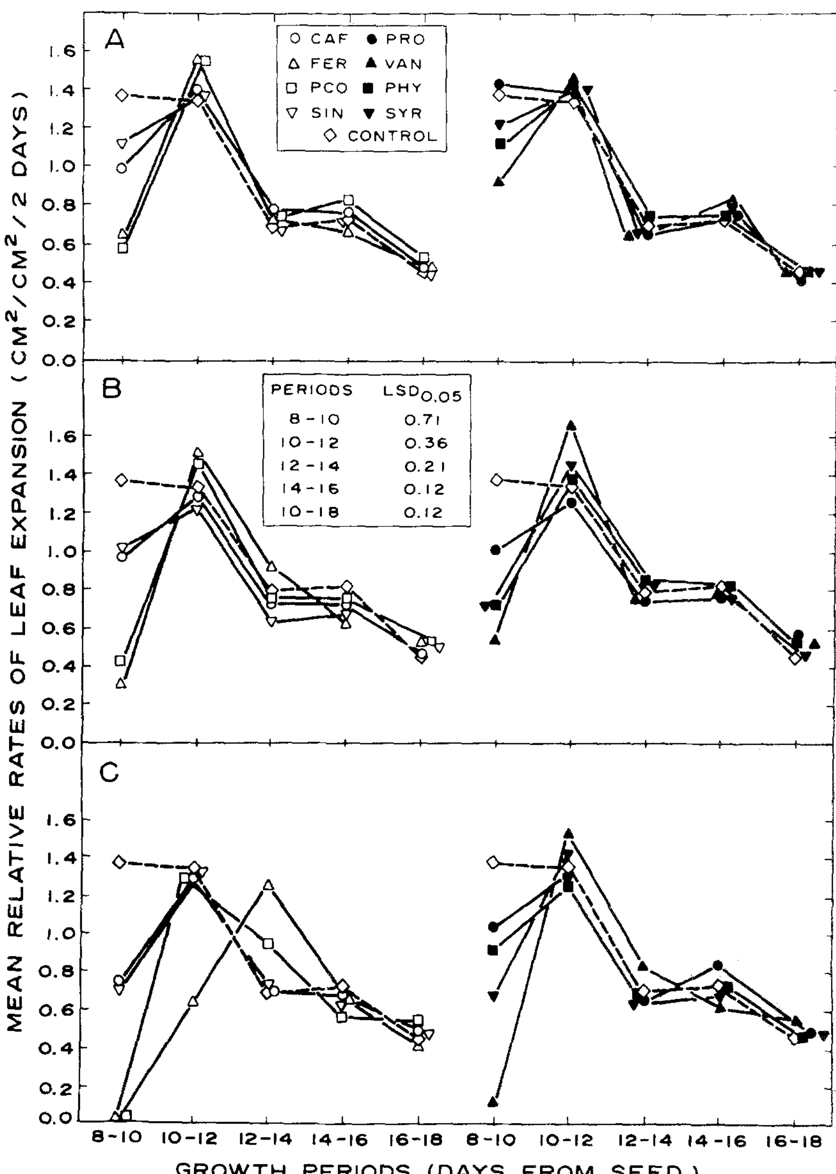


Fig. 2. Effects of multiple treatments of eight phenolic acids (A = 0.25 mM, B = 0.5 mM, C = 1.0 mM) on the mean relative rates of leaf expansion of cucumber seedlings. Treatments were initiated when seedlings were six days old. Statistical comparisons between means can be made only within each growth period. Points are connected only to aid in the visualization of patterns over time ($N = 3$).

TABLE 2. PARTIAL REGRESSION COEFFICIENTS AND $R^2$ VALUES FOR MEAN RELATIVE RATES OF LEAF AREA EXPANSION ($cm^2/cm^2/2$ DAYS) OF CUCUMBER SEEDLINGS TREATED WITH VARIOUS PHENOLIC ACIDS EVERY OTHER DAY FROM DAY 6[a]

| Phenolic acids | Growth period (days) | Intercept | Linear | Quadratic | $R^2$ |
|---|---|---|---|---|---|
| FER[b] | 8–10 | 1.146 | -1.282 | | 0.71 |
| | 10–12 | 1.344 | 1.387 | -2.078 | 0.85 |
| | 12–14 | 0.636 | 0.614 | | 0.88 |
| PCO | 8–10 | 1.138 | -1.246 | | 0.62 |
| | 12–14 | 0.690 | 0.241 | | 0.32 |
| | 14–16 | 0.742 | 0.277 | -0.457 | 0.56 |
| PRO | 14–16 | 0.725 | 0.093 | | 0.58 |
| SIN | 8–10 | 1.367 | -0.624 | | 0.53 |
| SYR | 8–10 | 1.329 | -0.716 | | 0.45 |
| VAN | 8–10 | 1.273 | -1.219 | | 0.73 |
| | 16–18 | 0.444 | 0.117 | | 0.54 |

[a]Concentrations ranged from 0 to 1 mM.
[b]FER = ferulic acid, PCO = *p*-coumaric acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.

acids every two days. Previously, we had concluded that the first ferulic acid treatment was a "shock" to cucumber seedling growth from which the seedlings rapidly recovered (Blum and Dalton, 1985). Within the time frame of our prior experiments, the "shock" and associated delay in growth could not be overcome by the treated plants, resulting in a reduction in leaf area and dry weight at harvest. A similar response was observed here, not only for ferulic acid, but also for the other seven phenolic acids. (Figure 1, Table 1). There was, however, an obvious difference in seedling response to at least two of the phenolic acids. Leaves visibly wilted for plants treated with 0.5 mM (or higher concentrations) of ferulic and *p*-coumaric acid, but not with the other phenolic acids at those concentrations. Osmotic differences associated with the increasing concentrations of ferulic acid were not responsible for the wilting of the leaves (Blum and Dalton, 1985), a conclusion further supported by the fact that the osmolalities of the eight phenolic acid solutions were similar.

*Phenolic Acid Mixtures.* Initially, leaf area was reduced by all eight phenolic acid treatments, but the significant reductions were rapidly lost for the *p*-hydroxybenzoic, protocatechuic, and sinapic acids once plants were returned to Hoagland's solution without phenolic acids (Tables 3 and 4). Table 4 presents

TABLE 3. LEAF AREA OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| Concentration of phenolic acids (mM) | Day from seed | Leaf area ($cm^2$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | CAF[b] | FER | PCO | PHY | PRO | SIN | SYR | VAN |
| 0 | | 65.10 | | | | | | | | |
| 0.5 | 15 | | 58.96 | 42.63 | 52.32 | 60.34 | 59.80 | 57.42 | 54.13 | 50.54 |
| 1.0 | | | 53.60 | 33.89 | 35.41 | 51.28 | 59.86 | 54.12 | 46.78 | 45.28 |
| 0 | | 113.65 | | | | | | | | |
| 0.5 | 17 | | 109.56 | 78.19 | 103.21 | 113.18 | 108.75 | 105.11 | 100.89 | 92.65 |
| 1.0 | | | 98.16 | 59.07 | 66.92 | 97.02 | 108.51 | 101.19 | 83.96 | 86.51 |
| 0 | | 164.98 | | | | | | | | |
| 0.5 | 19 | | 161.23 | 118.14 | 152.25 | 166.03 | 155.44 | 153.61 | 146.06 | 135.58 |
| 1.0 | | | 146.47 | 87.96 | 96.90 | 144.25 | 158.67 | 150.33 | 122.54 | 127.57 |
| | | | FER-CAF | | FER-PCO | FER-PHY | FER-PRO | FER-SIN | FER-SYR | FER-VAN |
| | 15 | | 35.84 | | 37.45 | 36.98 | 33.80 | 40.31 | 45.11 | 44.60 |
| 0.5 + 0.5 | 17 | | 67.56 | | 65.22 | 69.07 | 64.54 | 74.84 | 84.70 | 77.84 |
| | 19 | | 101.31 | | 97.22 | 99.15 | 99.88 | 110.99 | 128.10 | 114.81 |

[a]Seedlings were treated once with phenolic acid solution when 13 days old. Nutrient solutions were changed every other day.
[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.

partial regression coefficients of models for which the effects of both phenolic acids were significant. It also presents coefficients for ferulic acid alone, since its effects were significant for all models including those not present in the table. Dry weight effects at final harvest were observed only for *p*-coumaric, ferulic, and syringic acids. The effects of these phenolic acids on final harvest dry weight could be described by the following equations:

Ferulic-*p*-coumaric

$$\text{dry weight (g)} = 0.662 - 0.291\ (\text{FER}) - 0.213\ (\text{PCO}),$$

$$\alpha = 0.0001,\ R^2 = 0.80$$

TABLE 4. PARTIAL REGRESSION COEFFICIENTS AND $R^2$ VALUES FOR LEAF AREA ($cm^2$) OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| Phenolic acids | Day from seed | Intercept | Linear for ferulic acid | Linear for other phenolic acid | Quadratic for ferulic acid | Quadratic for other phenolic acid | Linear interaction | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| FER-CAF[b] | 15 | 63.71 | -34.21 | -11.92 | | | | 0.77 |
| | 17 | 113.40 | -60.46 | -16.64 | | | | 0.82 |
| | 19 | 165.73 | -85.58 | -21.55 | | | | 0.84 |
| FER-PCO | 15 | 63.38 | -30.91 | -25.82 | | | | 0.84 |
| | 17 | 113.06 | -58.86 | -10.41 | | -35.74 | | 0.89 |
| | 19 | 165.51 | -83.63 | -10.86 | | -57.75 | | 0.87 |
| FER-PHY | 15 | 64.25 | -34.31 | -13.32 | | | | 0.71 |
| FER-PRO | 15 | 65.44 | -69.63 | -7.72 | 38.07 | | | 0.91 |
| FER-SIN | 15 | 62.75 | -31.81 | -9.71 | | | | 0.81 |
| FER-SYR | 15 | 62.02 | -28.58 | -13.66 | | | | 0.73 |
| | 17 | 111.91 | -55.76 | -26.76 | | | 56.20 | 0.83 |
| | 19 | 163.25 | -78.28 | -39.45 | | | 94.85 | 0.76 |
| FER-VAN | 15 | 61.81 | -30.01 | -17.73 | | | 26.63 | 0.83 |
| | 17 | 113.65 | -87.27 | -56.84 | 32.69 | 29.70 | 82.60 | 0.91 |
| | 19 | 164.98 | -110.34 | -80.21 | 33.32 | 42.80 | 104.30 | 0.90 |
| FER | 15 | 62.81 | -31.21 | | | | | 0.90 |
| | 17 | 110.92 | -54.58 | | | | | 0.92 |
| | 19 | 162.21 | -77.02 | | | | | 0.93 |

[a]Seedlings were treated once with phenolic acid solutions when 13 days old. Nutrient solutions were changed every other day. Concentrations ranged from 0 to 1 mM. Mixtures contained 0.5 mM of each phenolic acid.

[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.

Ferulic–syringic

$$\text{dry weight (g)} = 0.637 - 0.252\ (\text{FER}) - 0.104\ (\text{SYR}),$$

$$\alpha = 0.0001,\ R^2 = 0.70$$

where FER, PCO, and SYR represent millimoles of ferulic, *p*-coumaric, and syringic acids, respectively.

In all cases, except ferulic–vanillic and ferulic–syringic acids, the inhibitory effects of the individual phenolic acids in the mixtures were additive in their effects on leaf area (Table 5). The inhibitory effects of ferulic-*p*-coumaric and ferulic–syringic acids were additive for dry weights. The linear interaction partial regression coefficients given in Table 4 were significant for leaf area of the ferulic–syringic and ferulic–vanillic acid mixtures. This indicates that the impact of these combinations on leaf area were not additive, but were, in fact, antagonistic (Table 5). Leaf area reductions by both mixtures were 16–25% less than expected. The 20% difference in leaf area at age 15 between the sum of the individual phenolic acids and the mixture of the ferulic–syringic acid treatment was not significant. Care must be taken in the interpretation of such effects, since we had noted earlier for cucumber radicle length that the magnitude of an antagonistic effect was concentration dependent (Blum et al., 1984). This aspect was addressed in more detail in a following experiment. Percent reductions given in the various tables (Table 5, etc.) were based on the actual data instead of the models to provide some indication of the range of variation that may be observed in such data. The models were used to test whether observed reductions in leaf area or mean relative rate of leaf expansion were additive. A significant interaction term in the model indicated that the effects were not additive (Blum et al., 1984).

The emphasis of this research was on leaf area instead of dry weight. We had previously determined that leaf area and seedling dry weight of cucumber were linearly correlated and that this relationship was not modified by nutrition and ferulic acid treatments (Blum and Dalton, 1985). Since leaf area can be easily obtained without destructive sampling and leaf area is a rapid indicator of plant response, we chose to utilize this seedling character for study. As in our previous study, the linear correlation coefficient for the full model (i.e., all phenolic acid treatments and concentrations, $r = 0.98$), the linear correlation coefficient after treatment effects were removed ($r = 0.93$), and the linear model for seedling leaf area and dry weight were highly significant (leaf area in $\text{cm}^2 = (-10.75) + (263.97)$ (dry weight in g), $\alpha = 0.0001$, $R^2 = 0.92$, $N = 72$).

Mean relative leaf expansion was reduced only for the two-day period (13–15) of the treatment (Tables 6 and 7). This short-term inhibition of growth was sufficient so that the effects on leaf area (Tables 3 and 4) and dry weight for

TABLE 5. PERCENT REDUCTION FOR LEAF AREA OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| | | Reduction (%) | | | | |
|---|---|---|---|---|---|---|
| Phenolic acids | Day from seed | Ferulic acid 0.5 mM | Other phenolic acid 0.5 mM | Sum | Mixture 0.5 + 0.5 mM | Sum − mixture |
| FER-CAF[b] | 15 | 34.52 | 9.43 | 43.95 | 44.95 | −1.00 |
| | 17 | 31.20 | 3.60 | 34.80 | 40.55 | −5.75 |
| | 19 | 28.29 | 2.27 | 30.66 | 38.59 | −7.93 |
| FER-PCO | 15 | | 19.63 | 54.15 | 42.47 | +11.68 |
| | 17 | | 9.19 | 40.39 | 42.61 | −2.22 |
| | 19 | | 7.72 | 36.01 | 41.07 | −5.06 |
| FER-PHY | 15 | | 7.31 | 41.83 | 43.20 | −1.37 |
| FER-PRO | 15 | | 8.14 | 42.66 | 48.08 | −5.42 |
| FER-SIN | 15 | | 11.80 | 46.32 | 38.08 | +8.24 |
| FER-SYR | 15 | | 16.85 | 51.37 | 30.71 | +20.66 |
| | 17 | | 11.23 | 42.43 | 25.47 | +16.96*[c] |
| | 19 | | 11.47 | 39.76 | 22.35 | +17.41* |
| FER-VAN | 15 | | 22.37 | 56.89 | 31.49 | +25.40* |
| | 17 | | 18.48 | 49.68 | 31.51 | +18.17* |
| | 19 | | 17.82 | 46.11 | 30.41 | +15.70* |

[a]Values derived from Table 3. Seedlings were treated once with phenolic acid solutions when 13 days old. Nutrient solutions were changed every other day.
[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.
[c]Significant level: $\leq 0.05$ level of probability.

seedlings treated with ferulic, *p*-coumaric, and syringic acids were still significantly reduced at final harvest. The effects of the phenolic acid mixture on leaf area (Tables 7 and 8) were lower than expected for the ferulic–syringic (24%) and ferulic–vanillic acid mixtures (20%).

Antagonistic effects of phenolic acid mixtures on radicle growth of cucumbers may vary with total concentration (Blum et al., 1984). To determine if this may also be the case for leaf expansion, mixtures of 0.125, 0.25, and 0.5 mM for several phenolic acids were tested. We tested the ferulic–*p*-coumaric acid mixture because its effects on mean relative rates of leaf expansion were additive and the ferulic–vanillic acid mixture because its effects were antagonistic (Tables

TABLE 6. MEAN RELATIVE RATES OF LEAF EXPANSION FOR CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS (GROWTH PERIOD 13–15)[a]

| Concentration phenolic acids (mM) | Mean relative rates ($cm^2/cm^2/2$ days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | CAF[b] | FER | PCO | PHY | PRO | SIN | SYR | VAN |
| 0 | 0.721 | | | | | | | | |
| 0.5 | | 0.631 | 0.318 | 0.519 | 0.626 | 0.699 | 0.612 | 0.598 | 0.570 |
| 1.0 | | 0.529 | 0.158 | 0.248 | 0.535 | 0.631 | 0.510 | 0.468 | 0.463 |
| | | FER-CAF | | FER-PCO | FER-PHY | FER-PRO | FER-SIN | FER-SYR | FER-VAN |
| 0.5 + 0.5 | | 0.292 | | 0.217 | 0.279 | 0.305 | 0.308 | 0.370 | 0.309 |

[a]Seedlings were treated once with phenolic acids when 13 days old. Nutrient solutions were changed every other day.

[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.

TABLE 7. PARTIAL REGRESSION COEFFICIENTS AND $R^2$ VALUES FOR MEAN RELATIVE RATES OF LEAF EXPANSION ($cm^2/cm^2$/2 DAYS) OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| Phenolic acids | Intercept | Linear for ferulic acid | Linear for other phenolic acid | Quadratic for ferulic acid | Quadratic for other phenolic acid | Linear interaction | $R^2$ |
|---|---|---|---|---|---|---|---|
| FER-CAF[b] | 0.708 | -0.899 | -0.163 | 0.349 | | | 0.98 |
| FER-PCO | 0.695 | -0.870 | -0.158 | 0.333 | -0.290 | | 0.96 |
| FER-PHY | 0.709 | -0.928 | -0.164 | 0.337 | | | 0.98 |
| FER-PRO | 0.722 | -1.002 | -0.077 | 0.438 | | | 0.98 |
| FER-SIN | 0.701 | -0.835 | -0.173 | 0.293 | | | 0.95 |
| FER-SYR | 0.680 | -0.562 | -0.116 | | -0.096 | 0.213 | 0.93 |
| FER-VAN | 0.713 | -1.024 | -0.258 | 0.470 | | 0.477 | 0.98 |

[a]Seedlings were treated once with phenolic acids when 13 days old. Nutrient solutions were changed every other day. Concentrations ranged from 0 to 1 mM. Mixtures contained 0.5 mM of each phenolic acid.
[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.

TABLE 8. PERCENT REDUCTION FOR MEAN RELATIVE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| | Reduction (%) | | | | |
|---|---|---|---|---|---|
| Phenolic acids | Ferulic acid 0.5 mM | Other phenolic acid 0.5 mM | Sum | Mixture 0.5 + 0.5 mM | Sum – mixture |
| FER-CAF[b] | 55.89 | 12.48 | 68.37 | 59.50 | +8.87 |
| FER-PCO | | 28.02 | 83.91 | 69.90 | +14.01 |
| FER-PHY | | 13.18 | 69.07 | 61.30 | +7.77 |
| FER-PRO | | 3.05 | 58.94 | 57.70 | +1.24 |
| FER-SIN | | 13.87 | 69.76 | 57.28 | +12.48 |
| FER-SYR | | 17.06 | 72.95 | 48.68 | +24.27*[c] |
| FER-VAN | | 20.94 | 76.83 | 57.14 | +19.69* |

[a]Values derived from Table 6. Seedling were treated once with phenolic acid solutions when 13 days old. Nutrient solutions were changed every other day.
[b]CAF = caffeic acid, FER = ferulic acid, PCO = *p*-coumaric acid, PHY = *p*-hydroxybenzoic acid, PRO = protocatechuic acid, SIN = sinapic acid, SYR = syringic acid, VAN = vanillic acid.
[c]Significant level: $\leqslant$0.05 level of probability.

7 and 8). The *p*-coumaric–vanillic acid mixture was also tested. All combinations were additive (Tables 9 and 10). In addition to the two-way mixtures we also included a three-way mixture of ferulic, *p*-coumaric, and vanillic acid. The effects of all three phenolic acids at concentrations of 0.167 and 0.333 mM were additive. The total concentration for the 0.333 mM mixture was 1 mM, a concentration sufficient for antagonistic effects in the previous experiment. The antagonistic effect for mean relative rate of leaf expansion observed previously (Table 8) for the ferulic–vanillic acid mixture at 0.5 mM was also absent (Table 10). This appeared to be due to the difference in the magnitude of inhibition by ferulic acid in the two experiments (56 vs. 27%). Einhellig and Eckrich (1984) observed that plant responses to ferulic acid were modified by temperature. This may have been the case here, since temperatures under the light banks varied with the ambient temperatures, and these experiments were carried out at different times. All these observations suggest that the presence or absence of antagonistic effects of individual phenolic acids in a mixture may or may not be related to the total concentration of a mixture, but are more likely to be related to the magnitude of effects (i.e., percent reduction) for each phenolic acid in

TABLE 9. MEAN RELATIVE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS (GROWTH PERIOD 14–16)[a]

| Concentration of phenolic acids (mM) | Mean relative rates ($cm^2/cm^2/2$ days) | | | |
|---|---|---|---|---|
| | FER[b] | PCO | VAN | |
| 0 | 0.647 | | | |
| 0.125 | 0.589 | 0.607 | 0.606 | |
| 0.25 | 0.547 | 0.543 | 0.584 | |
| 0.5 | 0.478 | 0.440 | 0.500 | |
| 0.75 | 0.284 | 0.410 | 0.432 | |
| 1.0 | 0.166 | 0.334 | 0.376 | |
| | FER-PCO | FER-VAN | PCO-VAN | FER-PCO-VAN |
| 0.125 + 0.125 | 0.465 | 0.554 | 0.580 | |
| 0.25 + 0.25 | 0.425 | 0.433 | 0.437 | |
| 0.5 + 0.5 | 0.294 | 0.325 | 0.382 | |
| 0.167 + 0.167 + 0.167 | | | | 0.428 |
| 0.333 + 0.333 + 0.333 | | | | 0.360 |

[a]Seedlings were treated once with phenolic acids when 14 days old. Nutrient solutions were changed every other day.
[b]FER = ferulic acid, PCO = *p*-coumaric acid, VAN = vanillic acid.

TABLE 10. PERCENT REDUCTION FOR MEAN RELATIVE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS TREATED ONCE WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| Phenolic acids | Concentration of phenolic acids | Reduction (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | FER | PCO | VAN | Sum | Mixture | Sum − mixture |
| FER-PCO[b] | 0.125 | 8.96 | 6.18 | | 15.14 | 28.13 | −12.99 |
| | 0.25 | 15.46 | 16.07 | | 31.53 | 34.31 | −2.78 |
| | 0.5 | 26.61 | 31.99 | | 58.60 | 54.56 | +4.04 |
| | 0.75 | 56.10 | 36.63 | | 92.73 | | |
| | 1.0 | 74.34 | 48.38 | | 122.72 | | |
| FER-VAN | 0.125 | 8.96 | | 6.34 | 15.30 | 14.37 | +0.93 |
| | 0.25 | 15.46 | | 9.74 | 25.20 | 33.08 | −7.88 |
| | 0.5 | 26.61 | | 22.72 | 49.33 | 49.77 | −0.44 |
| | 0.75 | 56.10 | | 33.23 | | | |
| | 1.0 | 74.34 | | 41.89 | | | |
| PCO-VAN | 0.125 | | 6.18 | 6.34 | 12.52 | 10.36 | +2.16 |
| | 0.25 | | 16.07 | 9.74 | 25.81 | 32.46 | −6.65 |
| | 0.5 | | 31.99 | 22.72 | 54.71 | 40.96 | +13.75 |
| | 0.75 | | 36.63 | 33.23 | | | |
| | 1.0 | | 48.38 | 41.89 | | | |
| FER-PCO-VAN | 0.167 | 7.99 | 8.11 | 6.73 | 22.83 | 22.83 (33.85)[c] | 0 (−11.02) |
| | 0.333 | 17.69 | 16.23 | 13.46 | 47.38 | 47.38 (44.36) | 0 (+3.02) |

[a]Values derived from Table 9. Seedlings were treated once with phenolic acids when 14 days old. Nutrient solutions were changed every other day.
[b]FER = ferulic acid, PCO = *p*-coumaric acid, VAN = vanillic acid.
[c]Numbers in parenthesis are based on actual values. Other values for three-way mixtures were based on models.

the mixture. The magnitude of effects for each phenolic acid in a mixture will be determined by its concentration, the susceptibility of the species, and the growth environment of the seedling (Einhellig and Eckrich, 1984; Blum and Dalton, 1985).

Since, in the previous experiment, leaves wilted when treated with 0.5 mM or higher concentrations of ferulic acid and *p*-coumaric acids, we also monitored water utilization (Table 11). In general, as the concentration of ferulic, *p*-coumaric, and vanillic acids increased, water utilization (ml/cm$^2$ of leaf/2 days) decreased. Effects on water utilization could be described by the following equation:

TABLE 11. WATER UTILIZATION FROM NUTRIENT CULTURE BY CUCUMBER SEEDLINGS TREATED WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS (GROWTH PERIOD 14–16)[a]

| Concentration of phenolic acids (mM) | Water utilization (ml/cm²/2 days) | | | |
|---|---|---|---|---|
| | FER[b] | PCO | VAN | |
| 0 | 0.71 | | | |
| 0.125 | 0.74 | 0.74 | 0.71 | |
| 0.25 | 0.65 | 0.60 | 0.67 | |
| 0.5 | 0.61 | 0.62 | 0.60 | |
| 0.75 | 0.36 | 0.59 | 0.62 | |
| 1.0 | 0.27 | 0.43 | 0.43 | |
| | FER-PCO | FER-VAN | PCO-VAN | FER-PCO-VAN |
| 0.125 + 0.125 | 0.53 | 0.66 | 0.65 | |
| 0.25 + 0.25 | 0.55 | 0.60 | 0.56 | |
| 0.5 + 0.5 | 0.26 | 0.41 | 0.50 | |
| 0.167 + 0.167 + 0.167 | | | | 0.47 |
| 0.333 + 0.333 + 0.333 | | | | 0.39 |

[a]Seedlings were treated once with phenolic acids when 14 days old. Nutrient solutions were changed every other day.
[b]FER = ferulic acid, PCO = *p*-coumaric acid, VAN = vanillic acid.

Ferulic acid

$$\text{ml/cm}^2\text{/2 days} = 0.766 - 0.484(\text{CONC}),\ \alpha = 0.0001,\ R^2 = 0.85$$

*p*-Coumaric acid

$$\text{ml/cm}^2\text{/2 days} = 0.723 - 0.250(\text{CONC}),\ \alpha = 0.0002,\ R^2 = 0.59$$

Vanillic acid

$$\text{ml/cm}^2\text{/2 days} = 0.731 - 0.248(\text{CONC}),\ \alpha = 0.0001,\ R^2 = 0.74$$

where CONC is in mM. The effects of mixtures on water utilization were additive except for the ferulic-*p*-coumaric acid mixture (Table 12), which was synergistic for the 0.125 and 0.5 mM treatments (i.e., the decline in water utilization was greater than expected).

*Transpiration.* Water loss by the first primary leaf was reduced within 24 hr after phenolic acid treatment for all but *p*-hydroxybenzoic acid (Figure 3) which took 48 hr. Normal transpiration rates were regained after three to six

TABLE 12. PERCENT REDUCTION OF WATER UTILIZATION FROM NUTRIENT CULTURE OF CUCUMBER SEEDLINGS TREATED WITH VARIOUS PHENOLIC ACIDS AND MIXTURES OF PHENOLIC ACIDS[a]

| Phenolic acids | Concentration of phenolic acid | Reduction (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | FER | PCO | VAN | Sum | Mixture | Sum − mixture |
| FER-PCO[b] | 0.125 | 0 | 0 | | 0 | 25.35 | -25.35*[c] |
| | 0.25 | 8.45 | 15.49 | | 23.94 | 22.53 | +1.41 |
| | 0.5 | 14.08 | 12.68 | | 26.76 | 63.38 | -36.62* |
| | 0.75 | 49.30 | 16.90 | | 66.20 | | |
| | 1.0 | 61.97 | 39.44 | | 101.41 | | |
| FER-VAN | 0.125 | 0 | | 0 | 0 | 7.04 | -7.04 |
| | 0.25 | 8.45 | | 5.63 | 14.08 | 15.49 | -1.41 |
| | 0.5 | 14.08 | | 15.49 | 29.57 | 42.25 | -12.68 |
| | 0.75 | 49.30 | | 12.68 | 61.98 | | |
| | 1.0 | 61.97 | | 39.44 | 101.41 | | |
| PCO-VAN | 0.125 | | 0 | 0 | 0 | 8.45 | -8.45 |
| | 0.25 | | 15.49 | 5.63 | 21.12 | 21.13 | -0.01 |
| | 0.5 | | 12.68 | 15.49 | 28.17 | 29.58 | -1.41 |
| | 0.75 | | 16.90 | 12.68 | 29.39 | | |
| | 1.0 | | 39.44 | 39.44 | 78.88 | | |
| FER-PCO-VAN | 0.167 | 6.46 | 13.90 | 12.07 | 32.43 | 32.45 (33.80)[d] | -0.02 (-1.37) |
| | 0.333 | 15.96 | 18.54 | 15.96 | 50.46 | 50.43 (45.07) | +0.03 (+5.39) |

[a]Values derived from Table 11. Seedlings were treated once with phenolic acids when 14 days old. Nutrient solutions were changed every other day.
[b]FER = ferulic acid, PCO = *p*-coumaric acid, VAN = vanillic acid.
[c]Significant level: $\leqslant 0.05$ level of probability.
[d]Numbers in parenthesis are based on actual values. Other values for three-way mixtures were based on models.

days for the single treatment. The second treatment delayed the recovery further. At day 16, the diffusive resistance (seconds/cm) for the control plants was 1.4, values for all other treatments ranged from 5.93 to 2.34. At day 22, diffusive resistance for control plants was 1.11. Values ranging from 2 to 5.15 were observed for the plants treated twice with *p*-coumaric (0.5 or 1.0 mM), *p*-hydroxybenzoic acid (0.5 or 1.0 mM), and ferulic acid (1.0 mM). Values for all other treatments were below 2. Phenolic acid alteration of stomatal function has also

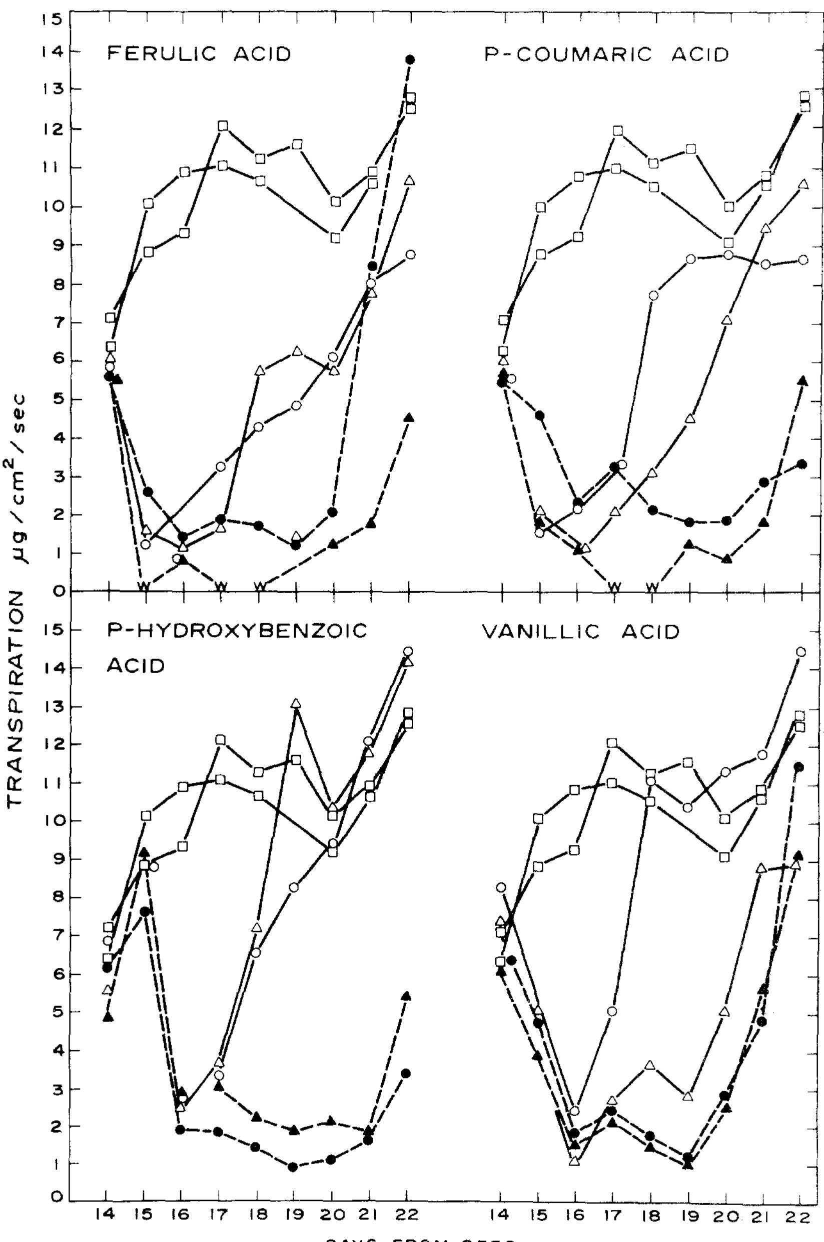


Fig. 3. Effects of ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids on transpiration of the first primary leaf of cucumber seedlings; □ = control seedlings (two control seedlings were measured), ○ = 0.5 mM treatment (day 14–16), △ = 1.0 mM treatment (day 14–16), ● = 0.5 mM treatments (day 14–16 and 16–18), and ▲ = 1.0 mM treatments (day 14–16 and 16–18).

been observed by Einhellig and Kuan (1971) and Einhellig and Muth (1980) for several other species.

## SUMMARY AND CONCLUSIONS

The data presented suggest that a number of phenolic acid microbial metabolic products of ferulic acid, as well as two other phenolic acids, *p*-coumaric and syringic acid, frequently found in soils, can reduce seedling dry weight, leaf area expansion, and water utilization of cucumber seedlings. However, the magnitude of effect of each of these phenolic acids varied. Water relations of cucumber plants appear to be involved, since the phenolic acid treatments resulted in stomatal closure. The fact that *p*-coumaric and ferulic acid caused visible wilting within hours after treatment suggested that water uptake by the roots was modified by these compounds. Recovery of the wilted leaves occurred usually within 48 hr. Transpiration rates recovered within three to six days after a single two-day treatment (Figure 3). The recovery of transpiration rates took even longer after two two-day phenolic acid treatments.

A persistent question in the area of allelopathic interactions has been whether available concentrations of inhibitors in soil environments are adequate to inhibit seedling growth. Estimates of individual phenolic acids in soils that are directly available to interact with plant roots generally are small—less than 0.1 mM (Whitehead et al., 1982). However, soils usually contain an array of phenolic acids (Whitehead, 1964; Whitehead et al., 1981, 1982). The data presented here indicate that cooperative activity among a number of compounds, each below the level of inhibition, can bring about an inhibitory effect if the total level is above that required for inhibition. We suspect that, in the majority of cases, in natural soils it is a combination of inhibitory substances that brings about an inhibitory effect instead of a single compound.

Further, the data indicate that growth effects on cucumber seedlings by the phenolic acids under study were a result of a "shock" and associated delay in growth whenever the threshold of inhibition was exceeded for only a short time period. In this series of experiments, that period was less than two days. Recovery of the growth rate, however, was rapid once the phenolic acids were removed from the root environment. Despite this recovery, however, signficant reductions in dry weight and leaf area were still evident after a week for several phenolic acids. This suggests that as long as cucumber seedlings are in a geometric growth phase and the delay in growth is sufficient, a complete recovery will not be possible until the growth rate of the treated plants exceeds that of the control plants. The greater the "shock" and associated delay, the higher the growth rate would have to be above that of the control plants once recovery started. For some of the phenolic acids tested, this did occur. The inhibitory effects of protocatechuic and sinapic acid on leaf area were lost immediately after the roots were placed into Hoagland's solution without these phenolic acids.

Since recovery rates appear to be delayed in poor nutrient environments (Blum and Dalton, 1985), such delays in growth may be even more important under such circumstances.

Finally, our data suggest that, at least for cucumber, episodic occurrences of phenolic acid concentrations (singly or in mixtures) above the threshold for inhibition may be an important factor in altering growth. If this is true under field conditions, then the timing of soil extractions to determine if phenolic acid concentrations are sufficient to bring about inhibition would appear to be critical in demonstrating allelopathic interactions.

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# ABSOLUTE CONFIGURATION OF MOSQUITO OVIPOSITION ATTRACTANT PHEROMONE, 6-ACETOXY-5-HEXADECANOLIDE

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**Abstract**—6-Acetoxy-5-hexadecanolide (Ia) in the oviposition attractant pheromone released from egg apical droplets of the mosquito *Culex pipiens fatigans* Wied. is shown to be the (−)-(5*R*,6*S*)- enantiomer. Identification was by chromatography of the 6-trifluoroacetoxy derivatives of the natural pheromone and of the synthetic (−)-(5*R*,6*S*)- (Ib) and (+)-(5*S*,6*R*)- (IIb) enantiomers on a capillary column having a chiral stationary phase comprising a derivative of (1*S*,3*S*)-chrysanthemic acid. The synthetic (−)-(5*R*,6*S*)- enantiomer (Ia) attracted oviposition of four fold more mosquito egg rafts than the control ($P < 0.01$) whereas for the (5*S*,6*R*)- enantiomer (IIa) there was no statistically significant oviposition attraction.

**Key Words**—Mosquito, *Culex pipiens fatigans*, Diptera, Culicidae, oviposition, attractant, pheromone, chiral chromatography, acetoxyhexadecanolide.

## INTRODUCTION

The mosquito *Culex pipiens fatigans* (= *quinquefasciatus*) Wiedemann and others of that genus are known to release an oviposition attractant from apical droplets that form on the eggs (Bruno and Laurence, 1979). *Cx. p. fatigans* is an important vector of filarial disease (elephantiasis), and it is possible that a synthetic oviposition attractant could be used for control if combined with chemical or mechanical means of destroying the eggs. The major component of the ovi-



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position attractant pheromone has recently been identified as *erythro*-6-acetoxy-5-hexadecanolide, but without examining its absolute configuration (Laurence and Pickett, 1982). The purpose of this study was to determine which of the enantiomers [5*R*,6*S*)- (Ia) or (5*S*,6*R*)- (IIa)] was present in the natural material.

## METHODS AND MATERIALS

*Compounds.* Racemic *erythro*-6-acetoxy-5-hexadecanolide (Ia + IIa) (Figure 1) was synthesized from *erythro*-5,6-dihydroxyhexadecanoic acid by treatment with acetic anhydride (Laurence and Pickett, 1982). Racemic *erythro*-6-acetoxy-5-dodecanolide (Ic + IIc) was prepared from the corresponding dihydroxydodecanoic acid in a similar manner (Laurence and Pickett, 1985). The (−)-(5*R*,6*S*)- (Ia) and (+)-(5*R*,6*S*)- (IIa) enantiomers were prepared in 98% optical purity via the Sharpless asymmetric epoxidation which gave appropriate stereochemistry at positions 5 and 6 (Mori and Otsuka, 1983). Trifluoroacetoxy derivatives [(Ib + IIb), (Id + IId), (Ib) and (IIb)] were prepared from the corresponding *erythro*-5,6-dihydroxycarboxylic acids (1 mg) by treatment with trifluoroacetic anhydride (100 μl) overnight in a sealed ampoule followed by evaporation of reagent under nitrogen. Trifluoroacetylation was confirmed by gas chromatography–mass spectrometry (GC-MS).

*Trifluoroacetylation of Natural Pheromone.* Apical dropletes from 20 egg rafts of *Cx. p. fatigans* (corresponding to ca. 6 μg of Ia + IIa) were removed into hexane (ca. 500 μl) using a fine glass rod (Laurence and Pickett, 1982). The hexane was removed under nitrogen, and the residue stored with 2 M NaOH

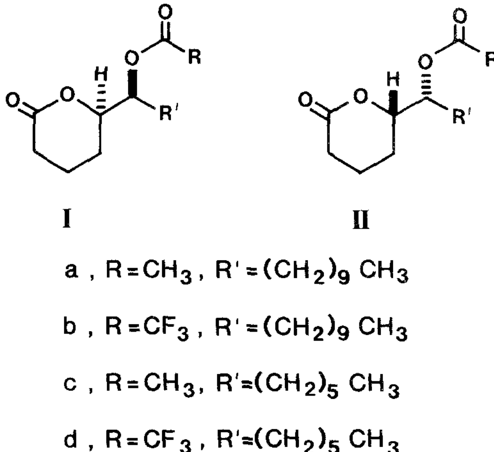


FIG. 1. Structures of mosquito pheromone, analogs, and derivatives.

FIG. 2. Structure of chiral GC phase.

(100 μl) overnight. the solution was then acidified (2 M HCl), extracted with ether (5 ml), dried over $MgSO_4 \cdot H_2O$, and the solvent evaporated under nitrogen. The residue was trifluoroacetylated by the technique described for the synthetic compounds.

*Gas chromatography-Mass Spectrometry (GC-MS).* GC employed a 25-m × 0.3-mm ID OV-101 bonded-phase, fused silica column (Scientific Glass Engineering) linked directly to a MM 70-70F mass spectrometer (V.G. Analytical) at 200°C and electron impact at 70 eV. The carrier gas was helium (235 mm/sec), and the oven temperature was held initially at 50°C for 10 min and then programed at 4°/min to 200°C: Id + IId, $R_t$ 50 min; Ib + IIb, $R_t$ 70 min.

*Chiral GC.* *O*-(1*S*,3*S*)-Chrysanthemoyl-(*R*)-mandelic acid (*S*)-1-(1′-naphthyl)ethylamide (III) (Figure 2) was prepared from (1*S*,3*S*)-chrysanthemic acid, (*R*)-mandelic acid, and (*S*)-1-(1′-naphthyl)ethylamine (Salford Ultrafine Chemicals and Research) by the method employed by Oi et al. (1983) for the enantiomer of this compound. This material was used to prepare a 25-m × 0.22-mm ID fused silica capillary column (Chrompak) which was mounted in a Pye series 104 gas chromatograph equipped with a flame ionization detector and with helium (15 psi) as the carrier gas. Samples were introduced using direct on-column injection onto a cooled column (25°C); after 20 sec the oven temperature was ballistically programed to 160°C, the maximum operating temperature of the column.

*Bioassay.* Hexane solutions (4 μl) containing either Ia or IIa (0.3 μg = ca. 1 egg raft equivalent) were placed on single polystyrene disks floating in bowls of water. The number of egg rafts laid overnight was determined for each enantiomer against a control employing hexane only. Each replicate experiment was a pair of oviposition bowls (treated and control) placed in a cage of mosquitoes which had fed on blood 72 hr previously. The bioassay protocol is more fully described by Laurence and Pickett (1985).

## RESULTS AND DISCUSSION

Chiral capillary columns based on trimethylsilylated valine separate some enantiomeric compounds (König, 1982) but failed to resolve racemic *erythro*-6-

acetoxy-5-hexadecanalide (Ia + IIa). The higher column temperatures, necessitated by the low volatility of this compound, were though to have prevented resolution. Therefore a lower-molecular-weight homolog (Ic + IIc) and its trifluoroacetoxy derivative (Id + IId) were examined, but these also were not resolved even at a temperature as low as 150°C. In an attempt to increase the interaction with the chiral column, the deacetyl derivatives and their carbamates, formed by reaction with isopropyl isocyanate (König et al., 1982), were examined but failed to chromatograph as discrete peaks.

Recently Oi et al.(1983) reported that a chiral column prepared from a derivative of (1*R*,3*R*)-chrysanthemic acid gave good resolution of chiral esters. We therefore investigated this type of column although, for convenience, the other enantiomer *O*-(1*S*, 3*S*)-chrysanthemoyl-(*R*)-mandelic acid (*S*)-1-(1′-naphthyl)ethylamide (III) was employed. Attempts to chromatograph racemic 6-acetoxy-5-hexadecanolide (Ia + IIa) or the homolog (Ic + IIc) again proved unsuccessful. However, the trifluoroacetoxy analog of the lower-molecular-weight compound (Id + IId) was resolved at 160°C ($R_t$ 86 and 88 min). At the same temperature, but with much longer retention times, the trifluoroacetoxy derivative of the synthetic racemate (Ib + IIb) corresponding to the pheromone showed almost baseline separation (Figure 3, trace A). Coinjection of this racemic material with the pure enantiomers Ib and IIb showed that the (5*R*,6*S*)-enantiomer eluted before the (5*S*,6*R*)-trifluoroacetoxyhexadecanolide. The trifluoroacetoxy derivative of the natural pheromone obtained from the egg raft apical droplets showed only one major component (trace B) which cochromatographed (trace A + B) with the first peak of the racemic mixture: the (5*R*,6*S*)-enantiomer.

The mass spectra from GC-MS of the synthetic trifluoroacetoxy derivatives (Ib + IIb), (Ib), and (IIb) were identical to that of the derivative from the natural pheromone which gave: *m/z* 366 ($M^+$, 7%), 252 ($M^+$-$CF_3CO \cdot OH$,14), 99($\overline{O:CC_4H_7:O}^+$, 100), 82(52), 81(70), 71(94), 69(67), 67(68), 55(90), 42(56). The lower-molecular-weight trifluoroacetoxy derivative (Id + IId) gave *m/z* 310 ($M^+$, 0.5%) 208 ($M^+$-$CF_3CO \cdot OH$,14) 99 ($\overline{O:CC_4H_7:O}^+$,100), 75(99), 55(96), 43(69), 42(72), 41(64), 29(79), 27(87). The interpretation is by analogy with accurate mass studies on the parent compound (Ia + IIa) (Laurence and Pickett, 1982). However, fragments formed by elimination of aldehyde, i.e., *m/z* 196 [$^{+}\cdot\overline{O \cdot CO \cdot (CH_2)_3C}HCO \cdot CF_3$] from the trifluoroacetoxy derivatives (Ib + IIb) and (Id + IId) were not as abundant (2% and 4%) as were the corresponding fragments from the parent compounds (Ia + IIa) (Laurence and Pickett, 1982) and (Ic + IIc) (Laurence and Pickett, 1985).

The synthetic (−)-(5*R* 6*S*)- (Ia) and (+)-(5*S*,6*R*)- (IIa) enantiomers were tested for activity at ca. 1 egg raft equivalent (0.3 $\mu$g) because this level of the racemic mixture gives a high response (81% of egg rafts laid in treated bowls) but is near the beginning of the steeply downward sloping part of the dose-

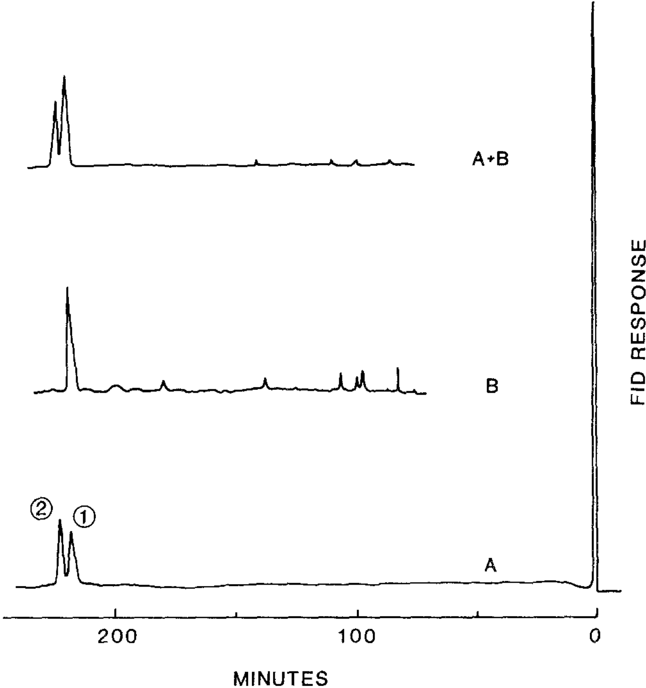


FIG. 3. Chiral chromatography of trifluoroacetoxy derivatives on (1*S*,3*S*)-chrysanthemic acid derivative III, 25m × 0.22 mm at 160°C: A, *erythro*-6-trifluoroacetoxy-5-hexadecanolide, (1) (5*R*,6*S*)- enantiomer (Ib), (2) (5*S*,6*R*)- enantiomer (IIb); B, derivative of natural pheromone; A + B, coinjection of A and B.

response curve and was therefore expected to allow maximum discrimination between samples of different activity (Laurence and Pickett, 1985). This was necessary because of the presence of 2% of the other enantiomer in each of the synthetic samples Ia and IIa. For the (−)-(5*R*,6*S*)- enantiomer (Ia), in 10 of the replicates more egg rafts were laid in the treated bowl, in none were there fewer, and in one there were equal numbers against the control (total egg rafts: in treated bowls, 70 (80%); in control bowls, 17; $t = 3.79$, $P < 0.01$). In contrast, for the (+)-(5*S*,6*R*)- enantiomer (IIa), in eight replicates more were laid in treated bowls, in three there were fewer, and in one there were equal numbers against the control (total egg rafts: in treated bowls, 221 (64%); in control bowls, 125; $t = 2.05$, $P > 0.05$).

Thus the absolute configuration of the major component of the oviposition attractant pheromone of *Cs. p. fatigans* is shown to be (−)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide (Ia), and it is the more biologically active enantiomer.

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# SEX PHEROMONES AND REPRODUCTIVE ISOLATION IN FOUR EUROPEAN SMALL ERMINE MOTHS[1]

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**Abstract**—Reproductive isolation among four sympatric small ermine moths (*Yponomeuta*) is analyzed in terms of niches in the sexual communication channel. Potential pheromone components were identified from pheromone gland secretions of *Y. evonymellus, Y. cagnagellus, Y. padellus,* and *Y. vigintipunctatus* by gas chromatography with flame ionization and electroantennographic detection and tested for behavioral activity in the field. The species were found to share (Z)-11-tetradecenyl acetate (Z11-14:OAc) in combination with varying proportions of the *E* isomer as primary sex pheromone components. *Y. cagnagellus* differs from the rest of the species by having only a small amount (1.5%) of *E* isomer relative to *Z* isomer in its pheromone. The closely related *Y. padellus* has a three-component pheromone including large amounts (ca. 400%) of (Z)-11-hexadecenyl acetate (Z11-16:OAc) in addition to Z11-14:OAc (100%) and *E*11-14:OAc (34%). Z11-16:OAc appears to reduce trap catches of *Y. evonymellus* and *Y. vigintipunctatus* when added to the pheromone. Although these species are the two most distantly related European small ermine moths, they seem to share the same sex pheromone, i.e., Z11-14:OAc (+20% *E*) and (Z)-11-tetradecenol. Our interpretation is that this might have been the sex pheromone of the ancestor of today's *Yponomeuta* species.

**Key Words**—*Yponomeuta*, Lepidoptera, Yponomeutidae, sex pheromone, reproductive isolation, gas chromatography, tetradecenyl acetate, tetradecenol, hexadecenyl acetate, ecological niches.

## INTRODUCTION

Small ermine moths of the genus *Yponomeuta* have a broad holarctic and paleotropical distribution (Herrebout et al., 1976). Nine forms occur sympatrically

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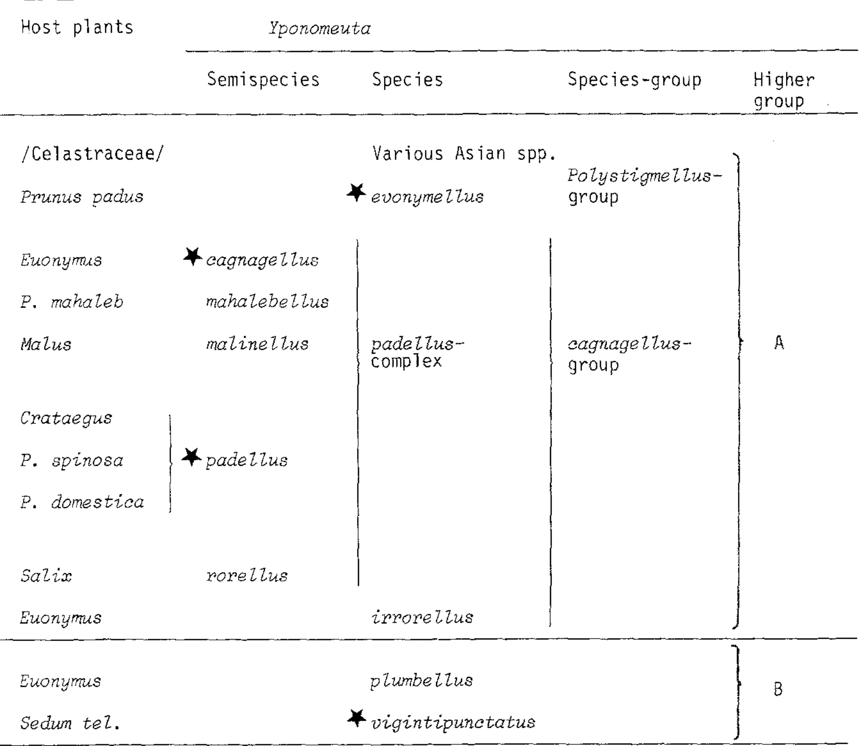

| Host plants | *Yponomeuta* Semispecies | Species | Species-group | Higher group |
|---|---|---|---|---|
| /Celastraceae/ | | Various Asian spp. | *Polystigmellus*-group | |
| *Prunus padus* | | ★ *evonymellus* | | |
| *Euonymus* | ★ *cagnagellus* | | | |
| *P. mahaleb* | *mahalebellus* | | | |
| *Malus* | *malinellus* | *padellus*-complex | *cagnagellus*-group | A |
| *Crataegus* | | | | |
| *P. spinosa* | ★ *padellus* | | | |
| *P. domestica* | | | | |
| *Salix* | *rorellus* | | | |
| *Euonymus* | | *irrorellus* | | |
| *Euonymus* | | *plumbellus* | | B |
| *Sedum tel.* | | ★ *vigintipunctatus* | | |

FIG. 1. European representatives of the genus *Yponomeuta* and their respective host plants after Gerrits-Heybroek et al. (1978); ★ = species analyzed in this study.

in the west palearctic region. Based on morphological characters, these belong to two species groups and are referred to as four distinct species and five semispecies (Figure 1), (Gerrits-Heybroek et al., 1978). The nine taxa show various degrees of similarity and are studied as steps in a speciation process (Wiebes, 1976; Menken, 1982). All taxa show considerable diel and seasonal overlap in their sexual activity (Hendrikse, 1979; Herrebout et al., 1976). If confined in cages the members of the "padellus-complex" hybridize and produce fertile offspring (Wildbolz and Riggenbach, 1965). A mechanistic explanation of their ability to maintain reproductive isolation under natural conditions might include differences in the sexual pheromone communication systems.

van der Pers (1982) investigated the pheromone sensilla on male ermine moth antenna by electrophysiological screening of 17 acetates and alcohols, earlier identified as or related to moth pheromone components. Almost all species responded to (*Z*)-11-tetradecenyl acetate (Z11-14 : OAc), (*E*)-11-tetradecenyl acetate (*E*11-14 : OAc), and (*Z*)-11-tetradecenol (Z11-14 : OH) with receptor cells showing a high degree of specificity. Behavioral studies with synthetic chemi-

cals in the laboratory as well as in the field also pointed in the direction of these compounds as pheromone components in several *Yponomeuta* species (Hendrikse et al., 1982).

Although a number of studies on reproductive isolation among moths by means of pheromones already are available, the interdisciplinary approach characteristic of the *Yponomeuta* project (Gerrits-Heybroek et al., 1978; Menken, 1982; Hendrikse et al., 1982; van der Pers, 1982) will provide extraordinary means for understanding the evolutionary processes involved. In the present study, pheromone gland secretions of *Y. evonymellus, Y. cagnagellus, Y. padellus,* and *Y. vigintipunctatus* occurring together in Southern Sweden, are analyzed. Pheromone candidates are tested in field-trapping experiments and potent synthetic pheromones are reported. The results are interpreted in terms of reproductive isolation among small ermine months.

## METHODS AND MATERIALS

*Insect Material.* In 1981 *Y. evonymellus* L., *Y. cagnagellus* Hübner, and *Y. padellus* L., collected in the field as pupae or late instar larvae, were obtained from Dr. W.M. Herrebout, Leiden, The Netherlands.

In 1982 and 1983 the same species were collected as late instar larvae or pupae in the vicinity of Lund, Sweden. The species identity was determined from pupal characteristics and the respective host plant and was later confirmed by examination of the adults. *Y. evonymellus* was collected on *Prunus padus* (L.), *Y. cagnagellus* on *Euonymus europeus* (L.), and *Y. padellus* on *Crataegus* sp. (L.), *Sorbus aucuparia* (L.), and *Prunus spinosa* (L.). Pupae were transferred to controlled environmental conditions to emerge or they were kept temporarily in a refrigerator at 4–10°C. *Y. vigintipunctatus* Retzius was obtained from Leiden, The Netherlands, in the autumn of 1982 as hibernating pupae. The pupae were stored at +4°C until the following spring. In 1983, second-generation, late instar larvae were collected at Hagestad in southern-most Sweden.

The adults emerged when exposed to approximately 20°C. Emerging pupae and adults were maintained at a reversed light–dark cycle (16 hr light–8 hr dark) and fed 5% sugar water upon emergence. The sexes were separated as pupae, and these and the emerging adults were kept in 250-ml plastic cups with a nylon screen cover, no more than eight individuals in each. Under these conditions most females readily displayed their pheromone glands in a typical calling posture 4–5 days after emergence.

*Collection of Pheromone.* In 1981 and 1982, extracts were prepared by soaking the ovipositor in pentane or hexane for 24 hr, as described by Löfstedt et al. (1982).

In 1983 a different procedure was used to collect the pheromone. When the

females were sexually active, the abdominal tips with the ovipositor extruded were dipped into heptane for about 15 sec. Comparison with a second extraction of the same material, carried out as described above, showed that quantitatively 90% of the pheromone components were recovered in the first wash and that the number of spurious and cuticular hydrocarbon peaks decreased substantially compared to the soaking method.

*Y. vigintipunctatus* calls in the middle of the scotophase, while the rest of the species are active at dawn (Hendrikse, 1979; and this study). Females used were 5–10 days old.

*Gas Chromatographic Analyses with Flame Ionization and Electroantennographic Detection.* Gas chromatographic (GC) analyses were performed on Hewlett Packard 5830 and 5880 gas chromatographs. For the simultaneous flame ionization and electroantennographic detection (FID and EAD) (Arn et al., 1975) an adjustable microneedle-valve effluent split (Scientific Glass Engineering, Inc.) was inserted and operated at a split ratio of 2:1.

Sample aliquots (1–5 μl) were injected splitless on WCOT fused silica capillary columns. The split valve was opened 0.5 min after injection, and the injector temperature was 225 or 250°C. Four different columns were used: two columns (0.19 mm ID × 20 and 50 m, respectively) coated with AT 1000 (Alltech Assoc.) in the laboratory, a commercially available polar BP 75 column (0.25 mm ID × 25 m) (Scientific Glass Engineering, Inc.) and a column (0.25 mm ID × 42 m) deactivated with 50% cyclic cyanosiloxane and coated with cross-linked 50% cyano-/25% tolysiloxane (Markides et al., 1983. Nitrogen carrier gas was supplied at 13 cm/sec linear flow (60°C) for the FID–EAD analyses. Hydrogen carrier was used for all of the other separations at a flow rate of 40 cm/sec (120°C).

Equivalent chain length (ECL) values were calculated relative to saturated straight-chain acetates for compounds of interest.

*Gas Chromatography-Mass Spectroscopy (GG-MS).* GC-MS analyses were performed in the EI mode on a Ribermag R 10-10c quadrupole GC-MS-DA system equipped with a Carlo Erba model 4160 GC. A 25-m fused silica column, 0.2 mm ID, coated with OV-101 (Ohio Valley Spec. Chem. Inc., Marietta, Ohio) nonpolar methyl silicone stationary phase was used for the separations. The helium carrier gas velocity was 23 cm/sec (60°C). A 1- to 3-μl sample was injected splitless at an injector temperature of 250°C and a column temperature of 60°C. The split valve was opened 50 sec after injection and, after 3 min, the column temperature was increased linearly by 8°C/min to 290°C. The electron energy used was 70 eV.

*Field Tests.* The attraction of male ermine moths to traps with test substances was investigated in southern Sweden in 1983. Experiments with *Y. vigintipunctatus* were carried out on grassland with abundant stands of *Sedum telephium* (L.) at Hagestad near Ystad. The rest of the experiments were per-

formed in the vicinity of Lund on pastureland with scrubs of *Crataegus, Euonymus,* and *Prunus spinosa* or along paths in a forest with rich abundance of *Prunus padus.* Compounds were dispensed from rubber septa (A.H. Thomas Company, Philadelphia, Pennsylvania) that were loaded by applying the compounds dissolved in hexane to the wide-end opening of the septum. The septa were placed in pheromone traps made in our laboratory (Löfqvist and Jönsson, unpublished) with a 4-cm opening in all directions and a 20 × 20-$cm^2$ sticky bottom. By placing the baits on metal pins in the ceiling, inside the trap, the bottoms could easily be replaced without touching the septa each time the traps were checked. This was a precaution to avoid contamination. Sticky wing traps (Albany International, Needham Heights, Massachusetts) were baited with two 4- to 10-day-old females confined in a small metal screen cylinder. The female traps were checked daily to make sure that the insects were in good condition.

The traps within one experimental series were spaced at least 10 m apart in the vegetation preferred by the respective species (see Figure 1). The traps in a replicate were frequently rotated to minimize positional effects.

*Chemicals.* Synthetic pheromone components were purchased from Dr. Simon Voerman, Institute for Pesticide Research, Wageningen, The Netherlands. These compounds were verified by GC to be more than 99% pure with respect to positional and geometric isomers.

## RESULTS

### GC-EAD Analysis and Preliminary Chemical Characterization

Exploratory GC-EAD analyses in 1981 with extracts of *Y. padellus* and *Y. cagnagellus* showed high EAD activity in the region of monounsaturated 14-carbon acetates, whereas the *Y. evonymellus* extracts caused no detectable activity. The retention time of the main EAD-active compound corresponded in both *Y. padellus* and *Y. cagnagellus* with that of synthetic *Z*11–14 : OAc on a 20-m AT 1000 capillary column. The amount present of the EAD-active compound was largest in *Y. cagnagellus* (approximately 3 ng/female. GC-MS analyses of two female equivalents (2 FE) of this extract on a non-polar OV-101 column revealed a compound with a fragmentation pattern and a retention time identical to that of *Z*11–14 : OAc. In the same way, tetradecyl acetate was identified as a gland constituent.

In spring 1983, some pupae of *Y. vigintipunctatus* also had become available for GC-EAD analysis. Analysis of a few female extracts on a polar BP 75 column, providing good resolution of *E*11- and *Z*11–14 : OAc, gave two EAD peaks with retention times identical to synthetic *E*11- and *Z*11–14 : OAc, respectively.

*GC-FID Analysis of Abdominal Tip Washes and Reference Compounds*

Abdominal tip washes from *Y. evonymellus* (Figure 2A), *Y. cagnagellus* (Figure 2B), *Y. padellus* (Figure 2C), and *Y. vigintipunctatus* were very similar when analyzed on the gas chromatograph. Reproducible peaks in the chromatograms were assigned the following structures based on retention times on two capillary columns of different polarity (Table 1): *Y. padellus* was found to contain 14:OAc, *E*11-14:OAc, Z11-14:OAc, 16:OAc, Z11-16:OAc, 14:OH, Z11-14:OH, and 16:OH. *Y. evonymellus* contained the same compounds except for Z11-16:OAc. *Y. cagnagellus extracts* showed peaks with retention times identical to 14:OAc, Z11-14:OAc, 16:OAc, 14:OH, Z11-14:OH, and 16:OH. Some *Y. cagnagellus* washes with high overall amounts of pheromone candidates also had a peak (height less than 2% of Z11-14: OAc) with the same ECL as *E*11-14: OAc when injected on the polar 50% cyano column.

*Y. vigintipunctatus* samples were not available for rigid comparison with synthetic compounds on the AT 1000 column. Analysis on the 50% cyano column revealed peaks with the same ECL as 14:OAc, *E*11-14:OAc, Z11-14:OAc, 16:OAc, 14:OH, and 16:OH. Analysis on the BP 75 column confirmed the assignment of 14:OAc, *E*11-14:OAc, Z11-14:OAc, and Z11-14:OH.

In all washes, except for those of *Y. padellus*, Z11-14:OAc was the major pheromone candidate identified in the samples. In *Y. padellus* the amount of Z11-16:OAc always exceeded that of the other compounds. The approximate composition of washes from the four species is given in Table 2. All species contain 3–4 ng of Z11-14:OAc per female, but a varying proportion of *E* isomer. In *Y. cagnagellus* the *E* isomer titer is zero or close to zero, but in *Y. padellus* it is as much as 34% of the Z11-14:OAc content.

*Exploratory Field Experiments*

Three series of baits, based on the preliminary chemical analyses were tested for *Y. evonymellus*, *Y. cagnagellus*, and *Y. padellus*, respectively. For *Y. evonymellus* Z11-14:OAc was tested alone at the 100-μg level, as well as in combination with 10 or 100 μg Z11-14:OH. No specimens were trapped (July 14–25), in spite of the high numbers of *Y. evonymellus* observed. The first experiment with *Y. cagnagellus* was carried out between July 18 and 25, with a series of dosages (10, 100, and 1000 μg) of Z11-14:OAc with 8% *E* isomer added. Some *Y. cagnagellus* as well as *Y. evonymellus* were trapped in insignificant numbers (1–3/trap). For *Y. padellus* 100:60 mixtures of Z11- and *E*11-14:OAc were tested at 10-, 100-, and 1000-μg dosages between July 13 and 23. Although adult *Y. padellus* were abundant in the area, none were trapped.

*Field Experiments with New Baits*

New series of baits based on the chemical analyses from 1983 were formulated and tested at the end of July.

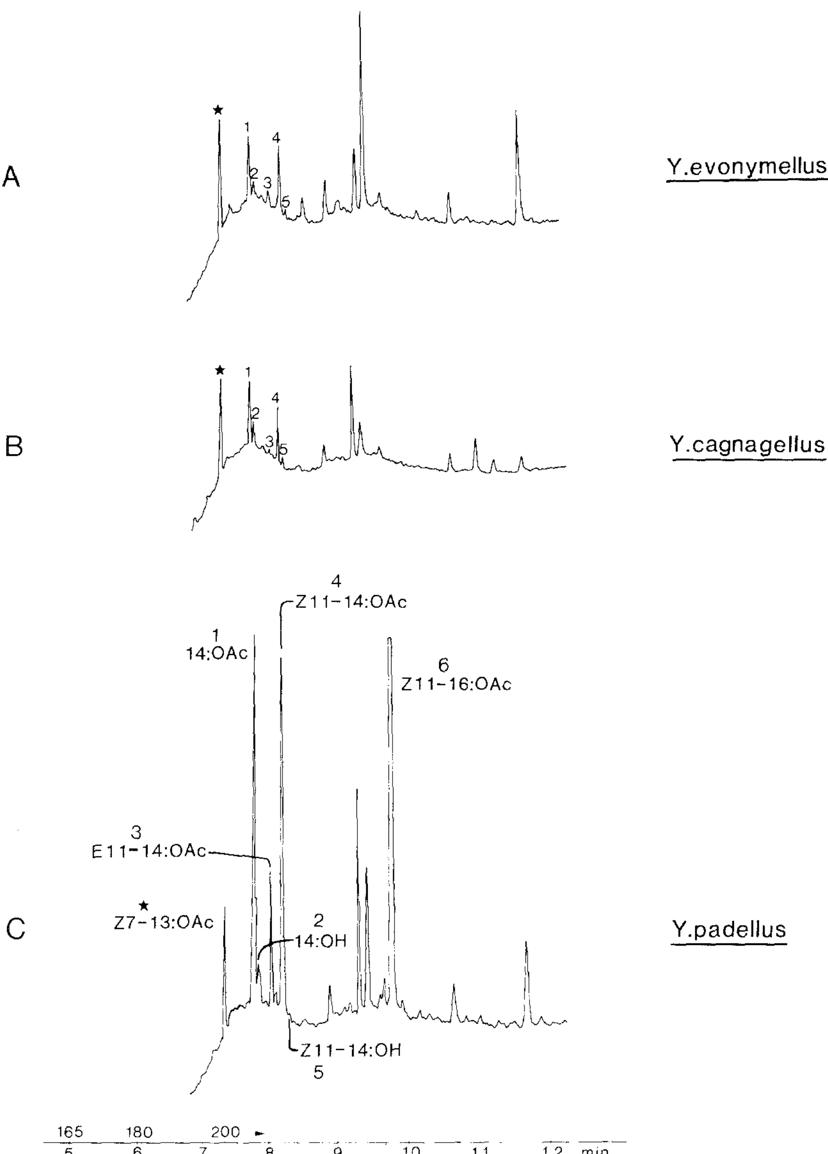


FIG. 2. Gas chromatograms from analyses of individual abdominal tip washes from three *Yponomeuta* species: *Y. evonymellus* (A), *Y. cagnagellus* (B), and *Y. padellus* (C). To each sample, 3 ng of the internal standard (*Z*)-7-tridecenyl acetate (*Z*7–13 : OAc) was added.

TABLE 1. EQUIVALENT CHAIN-LENGTH VALUES OF PHEROMONE COMPONENT CANDIDATES AND SYNTHETIC REFERENCES ON GC COLUMNS OF DIFFERENT POLARITY[a] USED FOR IDENTIFICATION

| | | | Species | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Synthetic reference | | *Y. evonymellus* | | *Y. cagnagellus* | | *Y. padellus* | | *Y. vigintipunctatus* | |
| Component | AT1000 | 50% cyano | AT1000 | 50% cyano | AT1000 | 50% cyano | AT1000 | 50% cyano | BP 75[b] | 50% cyano |
| 14:OAc | 1400 | 1400 | 1400 | 1400 | 1400 | 1400 | 1400 | 1400 | 1400(1400) | 1400 |
| *E*9–14:OAc | 1433 | 1431 | – | – | – | – | – | – | – (1432) | – |
| *Z*9–14:OAc | 1440 | 1447 | – | – | – | – | – | – | – (1441) | – |
| *E*11–14:OAc | 1441 | 1438 | 1441 | 1438 | *c* | *c* | 1440 | 1438 | 1444(1443) | 1438 |
| *Z*11–14:OAc | 1456 | 1459 | 1454 | 1460 | 1455 | 1459 | 1455 | 1460 | 1457(1457) | 1460 |
| 16:OAc | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | *d* (1600) | 1600 |
| *Z*11–16:OAc | 1638 | 1650 | – | – | – | – | 1639 | 1649 | *d* (1639) | – |
| 14:OH | 1466 | 1410 | 1467 | 1411 | 1466 | 1410 | 1467 | 1410 | ? | 1410 |
| *Z*11–14:OH | 1525 | 1470 | 1525 | 1472 | 1525 | 1471 | 1525 | 1471 | 1545(1545) | 1471 |
| 16:OH | 1667 | 1612 | 1668 | 1614 | 1668 | 1615 | 1668 | 1614 | ? | ? |

[a] – = no detectable peak; ? = no data available.
[b] No *Y. vigintipunctatus* extracts available for analysis on AT1000. Rectified by analysis on BP 75. ECL values of synthetic standards given in parentheses.
[c] Minute peak with approximately corresponding ECL present in some extracts, but not picked up by the integrator.
[d] If present, hidden by tailing peak eluting prior to the compound of interest.

TABLE 2. RELATIVE AMOUNTS OF ALIPHATIC ACETATES AND ALCOHOLS IN FOUR SPECIES OF ERMINE MOTHS (*Yponomeuta*)[a]

| | *Y. evonymellus*[b] | *Y. cagnagellus*[b] | *Y. padellus*[b] | *Y. vigintipunctatus*[c] |
|---|---|---|---|---|
| 14:OAc | 57 | 37 | 35 | 17 |
| 14:OH | 10 | 22 | 8 | ?[e] |
| *E*11-14:OAc | 24 | ≤1.5 | 34 | 21 |
| *Z*11-14:OAc[d] | 100 (2.7 ng) | 100 (4 ng) | 100 (3.3 ng) | 100 (~3 ng) |
| *Z*11-14:OH | 21 | 22 | 18 | 28 |
| 16:OAc | 44 | 65 | 82 | ?[f] |
| 16:OH | 18 | 85 | 20 | ?[e] |
| *Z*11-16:OAc | 0 | 0 | 277 | ?[f] |

[a]*Z*11-14:OAc = 100 in all species.
[b]Sample from a batch of four females analyzed on 50% cyano column.
[c]Sample from a batch of twenty females analyzed on BP 75 column.
[d]Absolute amount in parentheses.
[e]No data collected.
[f]Not resolved from preceding peak.

*Y. evonymellus*. *Z*11-14:OAc (100 μg) with the addition of *E*11-14:OAc (20%) was attractive, but *Z*11-14:OH was also required to obtain the same catches as with virgin females of *Y. evonymellus* (Table 3).

*Y. cagnagellus*. A small amount of *E*11-14:OAc (1.5%) relative to *Z*11-14:OAc seemed to be a critical key to attractivity (Table 4). Identification of trapped specimens proved to be a difficult task. The traps were dispensed in spindle trees (*Euonymus*), where we had observed larval infestations of *Y. cag-*

TABLE 3. TRAP CATCHES OF MALE *Y. evonymellus* WITH VIRGIN FEMALES AND VARIOUS COMBINATIONS OF SYNTHETIC PHEROMONE COMPONENTS, JULY 1983

| Stimulus (μg) | Males/trap ($\bar{X}$ ± SEM)[a] |
|---|---|
| *Z*11-14:OAc/*E*11-14:OAc/*Z*11-14:OH | |
| 100:0:0 | 0.0 ± 0.0 |
| 100:20:0 | 5.8 ± 1.3a |
| 100:20:20 | 26.4 ± 5.1b |
| 2 virgin females | 34.2 ± 20.0b |
| Unbaited | 0.2 ± 0.1 |

[a]Six replicates with female baited traps, nine for the rest of the stimuli. Numbers followed by the same letter are not significantly different by analysis of variance ($\sqrt{X + 0.5}$ transformation) followed by Duncan's multiple range test ($P < 0.05$). Numbers with no letter are not included in the statistical test.

TABLE 4. TRAP CATCHES OF MALE *Y. cagnagellus* WITH VARIOUS COMBINATIONS OF SYNTHETIC PHEROMONE COMPONENTS, JULY-AUGUST 1983

| Stimulus | Males/trap ($\bar{X}$ ± SEM)[a] |
|---|---|
| Unbaited | 0.0 ± 0.0 |
| *Z*11-14:OAc 100μg | 0.0 ± 0.0 |
| *Z*11-14:OAc 100μg + | |
| *Z*11-14:OH 20μg | 0.2 ± 0.2a |
| *E*11-14:OAc 8μg | 2.8 ± 1.1ab |
| *E*11-14:OAc 1.5μg | 10.8 ± 5.3bc |
| *E*11-14:OAc 1.5μg + *Z*11-14:OH 20μg | 17.8 ± 7.9c |
| *Z*11-14:OH 20μg + *Z*11-16:OAc 3μg | 0.4 ± 0.2a |

[a]Five replicates. Numbers followed by the same letter are not significantly different by analysis of variance ($\sqrt{X + 0.5}$ transformation) followed by Duncan's multiple range test ($P < 0.05$). Numbers with no letter are not included in the statistical test.

*nagellus* (identified from host plant preference and from collected insects, emerged at the laboratory). At present we cannot distinguish *Y. cagnagellus* positively from the very similar *Y. malinellus*, when caught in sticky traps, but no infestations of *Y. malinellus* had been observed in the trapping area.

*Y. padellus*. A blend of Z11-14:OAc, *E*11-14:OAc, and Z11-16:OAc in the female ratio (100:34:400 μg) trapped as many male *Y. padellus* as virgin females did (Table 5). The obligate three-component character of the *Y. padellus* pheromone was corroborated in a parallel test where the following four baits were found to be inactive (i.e., no *Y. padellus* trapped):Z11-14:OAc (100 μg), Z11-16:OAc (100 μg), Z11-14:OAc/Z11-16:OAc (100:100μg), and Z11-14:OAc/Z11-16:OAc (100:400 μg).

*Additional Experiments with* Y. evonymellus *and* Y. padellus. Additional experiments were carried out with *Y. evonymellus* and *Y. padellus* to determine the trapping activity of additional compounds found, or possibly present, in the extracts.

In *Y. evonymellus*, addition of 14:OAc and 14:OH did boost the activity over the three-component blend, while addition of Z11-16:OAc reduced the trap catch to zero (Table 6).

The following compounds were added to the *Y. padellus* mixture (100 μg Z11-14:OAc/34 μg *E*11-14:OAc/400 μg Z11-16:OAc) that trapped 25.8 ± 6.3 males (mean ± SEM) without any statistically significant increase in the trap catches: 20 μg Z11-14:OH (12.2 ± 3.2), 20 μg Z11-14:OH + 15 μg *E*11-14:OH (7.9 ± 3.2), 35 μg 14:OAc + 8 μg 14:OH + 20 μg Z11-14:OH (11.0 ± 3.5), and 35 μg 14:OAc + 8 μg 14:OH + 20 μg Z11-14:OH + 15 μg *E*11-14:OH (9.7 ± 3.4). ($N = 6$, one-way ANOVA on $\sqrt{X + 0.5}$ transformations,

TABLE 5. TRAP CATCHES OF MALE *Y. padellus* WITH VIRGIN FEMALES AND VARIOUS COMBINATIONS OF SYNTHETIC PHEROMONE COMPONENTS, JULY 1983

| Stimulus (μg) | Males/trap ($\bar{X}$ ± SEM)[a] |
|---|---|
| *Z*11-14:OAc/*E*11-14:OAc/*Z*11-16:OAc | |
| 100:34:0 | 0.6 ± 0.2a |
| 100:60:0 | 0.3 ± 0.2 |
| 100:34:400 | 24.7 ± 4.1b |
| 2 virgin females | 17.9 ± 4.4b |
| Unbaited | 0.0 ± 0.0 |

[a]Six replicates. Numbers followed by the same letter are not significantly different by analysis of variance ($\sqrt{X + 0.5}$ transformation) followed by Duncan's multiple range test ($P < 0.05$). Numbers with no letter are not included in the statistical test.

$F = 1.82$; $0.25 > P > 0.10$). The tendency was rather a decrease in attractivity, when the additional compounds were added.

In *Y. evonymellus*, a bait with a relative amount of Z11-14:OH lower than in females was the most attractive (Figure 3). The exact amount of Z11-16:OAc added to the Z11/*E*11-14:OAc blend in *Y. padellus* did not seem critical to the attraction (Figure 3), but a maximum seemed to occur with the natural ratio between the tetradecenyl acetates and the hexadecenyl acetate.

*Y. vigintipunctatus*. Pure Z11-14:OAc was unattractive to *Y. vigintipunctatus*. However, high catches were obtained in traps with 20% *E*11-14:OAc added (Table 7, August). A first experiment did not allow discrimination be-

TABLE 6. TRAP CATCHES OF MALE *Y. evonymellus* WITH *Z*11-14:OAc (100 μg), *E*11-14:OAc (20 μg) AND *Z*11-14:OH (20 μg) PLUS ADDITIONAL COMPONENTS, JULY-AUGUST 1983

| Stimulus | Males/trap ($\bar{X}$ ± SEM)[a] |
|---|---|
| *Z*11-14:OAc (100 μg) + *E*11-14:OAc (20 μg) + *Z*11-14:OH (20 μg) | |
| + 0 | 9.3 ± 3.0a |
| + *E*11-14:OH (5 μg) | 6.3 ± 2.2a |
| + 14:OAc (60 μg) + 14:OH (10 μg) | 33.7 ± 6.3b |
| + *Z*11-16:OAc (400 μg) | 0.0 ± 0.0 |

[a]Three replicates. Numbers followed by the same letter are not significantly different by analysis of variance ($\sqrt{X + 0.5}$ transformation) followed by Duncan's multiple range test ($P < 0.05$). Numbers with no letter not included in the statistical test.

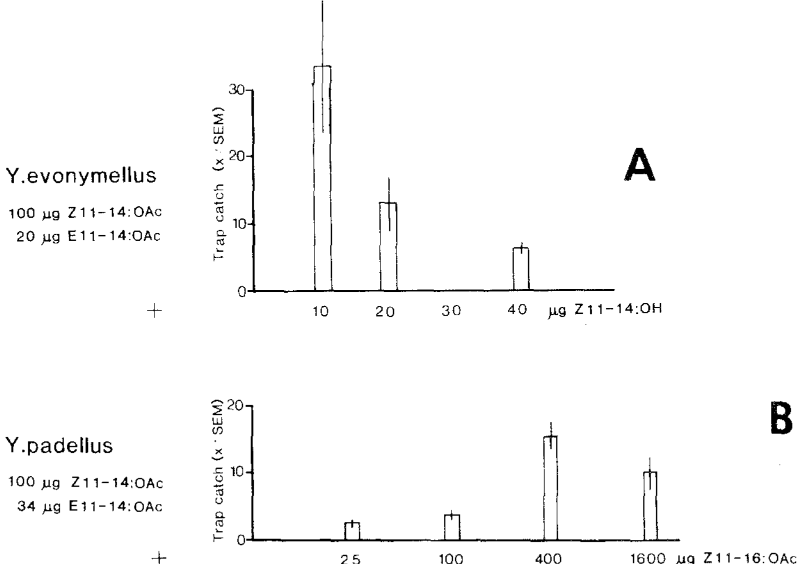


FIG. 3. Average trap catches of male *Y. evonymellus* ($n$ = 3) (A) and *Y. padellus* ($n$ = 6) (B) with various amounts of the third component (Z11-14:OH and Z11-16:OAc, respectively) added to the Z11/*E*11-14:OAc blend.

tween this two-component blend and baits with Z11-14:OH and/or 14:OAc added. A second experiment (Table 7, September) was completed towards the end of the flight period, with much lower overall trap catches. In this experiment, both 14 : OAc and Z11–14 : OH increased the trap catch over the two-component blend, whereas Z11–16 : OAc was found to reduce the trap catch.

*Z/E Ratios*

Experiments in which the Z11/*E*11–14 : OAc ratio was varied over a wide range were carried out to investigate the contribution of different isomer ratios to the reproductive isolation among the ermine moths (Figure 4). We were not able to collect replicates of *Y. evonymellus* because of the low population level towards the end of the flight period. However, the total trap catches indicate that the males are insensitive to changes ($\pm$10%) in the *Z/E* ratio. This is also the case in *Y. padellus*. In *Y. cagnagellus* a large amount of the *E* isomer lowers the attraction of males, and in *Y. virgintipunctatus* maximal attraction occurs to the ratio produced by the females.

DISCUSSION

This study shows that Z11–14 : OAc is a major pheromone component in four European ermine moths. However, this component alone was not attractive

TABLE 7. TRAP CATCHES OF MALE *Y. vigintipunctatus* IN TWO EXPERIMENTS WITH VARIOUS COMBINATIONS OF SYNTHETIC PHEROMONE COMPONENTS

| Stimulus | Males/trap ($\bar{X}$ ± SEM)[a] |
|---|---|
| August | |
| Unbaited | 0.0 ± 0.0 |
| *Z*11-14:OAc 100 μg | 0.3 ± 0.3 |
| *Z*11-14:OAc 100 μg + | |
| *E*11-14:OAc 20 μg | 122.8 ± 12.7a |
| *E*11-14:OAc 20 μg + *Z*11-14:OH 50 μg | 60.8 ± 15.1a |
| *E*11-14:OAc 20 μg + *Z*11-14:OH 50 μg | |
| + 14:OAc 15 μg | 85.3 ± 36.2a |
| September | |
| *Z*11-14:OAc 100 μg + *E*11-14:OAc 20 μg | 3.0 ± 1.6a |
| *Z*11-14:OAc 100 μg + *E*11-14:OAc 20 μg + | |
| 14:OAc 15 μg | 9.8 ± 4.1b |
| *Z*11-14:OH 10 μg | 33.8 ± 10.8b |
| *Z*11-16:OAc 400 μg | 0.5 ± 0.3 |

[a]Four replicates. Numbers within each experiment followed by the same letter are not significantly different by analysis of variance ($\sqrt{X + 0.5}$ transformation) followed by Duncan's multiple range test ($P < 0.05$). Numbers with no letter not included in the statistical test.

to any of the species in field experiments. Various amounts (1.5–36%) of *E*11–14:OAc and, in all but one species (*Y. cagnagellus*), a third component were necessary to achieve the highest trap catches. The amount of the third component relative to the Δ11–14:OAc isomers was not critical in any of the species, but a wide range of blends trapped high numbers of males. The tendency of baits with relative amounts of alcohol lower than found in the insects to be the most attractive in both *Y. evonymellus* and *Y. vigintipunctatus* could be due to the significantly higher release rates of alcohols from rubber septa compared to those of the corresponding acetates (Butler and McDonough, 1979, 1981). The relative release rates of alcohols from rubber septa compared to those of acetates might be higher than from female glands.

As can be expected with a synthetic pheromone, the three-component mixtures for *Y. evonymellus* and *Y. padellus* were competitive with virgin females. No such comparison was possible in *Y. cagnagellus* and *Y. vigintipunctatus* due to the lack of newly emerged females available for the trapping period.

Three saturated compounds with pheromone-like structure were detected in the gland extracts of all the species. The amounts of 14:OAc and 16:OAc were appreciable and 14:OH also occurred consistently. In *Y. evonymellus* and *Y. vigintipunctatus*, 14:OAc seemed to be behaviorally active, although the ex-

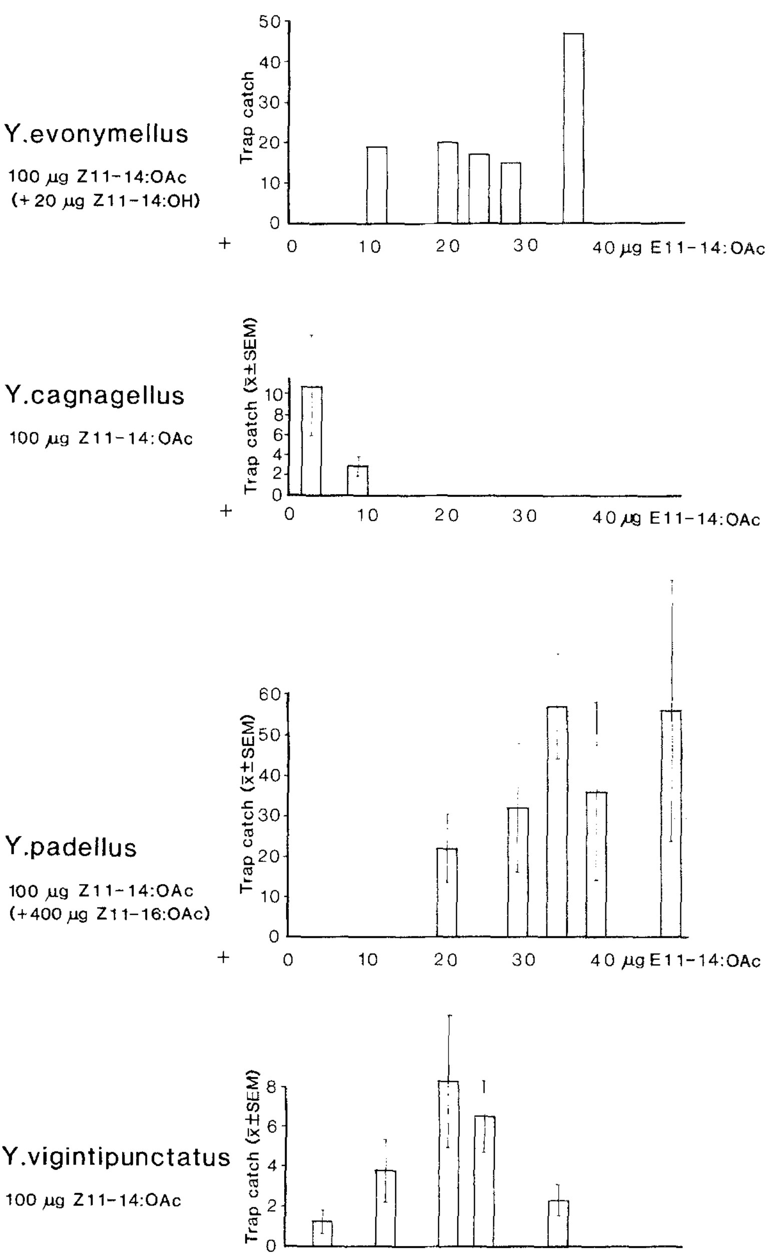


FIG. 4. Average male trap catches in four *Yponomeuta species*, with various *Z*11/*E*11-14:OAc ratios, but with the amount of the third components held constant: *Y. evonymellus*, *Y. cagnagellus* ($n = 5$), *Y. padellus* ($n = 3$) and *Y. vigintipunctatus* ($n = 4$).

periments were not conclusive. Subsequent experiments have confirmed the activity of 14:OAc in *Y. evonymellus* and *Y. Vigintipunctatus,* as well as in *Y. cagnagellus (Löfstedt, unpublished). As in many other months (Bjostad and Roelofs, 1983; Roelofs and Brown, 1982), the pheromone components found should be derived from palmitic acid, by a chain-shortening enzyme and a delta-11 desaturase working in combination. thus the common* Z11- and *E* 11-tetradecenyl moieties can be derived from chain-shortening of hexadecanoate to tetradecanoate with subsequent △11-desaturation and reduction. In *Y. padellus,* the delta-11 desaturase also seems to work on the hexadecanoate, producing (*Z*)-11-hexadecenoate. This precursor would then be reduced and acylated to produce *Z*11-16:OAc.

Our poor understanding of the reception of multicomponent pheromones sets a certain limit to the predictive value of electrophysiological screening in pheromone identification. The single sensillum investigation of male ermine moth antennae in all species dealt with in this study (van der Pers, 1982) revealed two cells in each sensillum, tuned to Z11–14 : OAc and *E*11–14 : OAc, respectively. In *Y. padellus,* the Z11–14 : OAc cell was also activated by Z11–16 : OAc and Z11–14 : OH. The alcohol activated the Z11–14 : OAc cell in the other species as well. In *Y. cagnagellus,* the *E*11–14 : OAc cell was activated to some extent by Z11–16 : OAc. To conclude, the response of the Z11–14 : OAc cell to Z11–16 : OAc in *Y. padellus* does match our finding that this compound is a pheromone component in the species. In contrast the *E*11–14 : OAc cell in *Y. cagnagellus* responded to not only Z11–16 : OAc as a potential pheromone constituent but also to half a dozen other compounds. Whether the sensitivity of the large spike amplitude cell to *Z*11-14:OAc, *Z*11-14:OH, and *Z*11-16:OAc in *Y. padellus* is due to a lack of absolute specificity or to separate acceptor sites on the same receptor cell tuned to the respective molecules should be investigated.

Greenfield and Karandinos (1979) applied the ecological niche concept to reproductive isolation. Following this approach, we treat the Z11/*E*11–14 : OAc ratio as one niche dimension in the "sexual communication channel" of ermine moths. Qualitative chemical differences (additional compounds, e.g., Z11–16 : OAc), and temporal factors (diel and seasonal activity differences) are additional dimensions. Differences in a single chemical niche dimension do not explain the reproductive isolation among the ermine moths. On the other hand, reproductive isolation always seems to be achieved when two or more dimensions are considered (Figure 5). Except for *Y. cagnagellus,* the isolation from other species is not determined by a specific isomer ratio, but by a specific third component or a temporal factor. In the *Y. padellus* pheromone, Z11–16 : OAc is an obligate component and, furthermore, it seems to reduce the attraction of *Y. evonymellus* and *Y. vigintipunctatus* males. W.M. Herrebout (personal communication) found that Z11–16 : OAc lowered trap catches of *Y. cagnagellus* when presented with virgin females. He also found evidence that increasing amounts of *E*11–14 : OAc added to Z11–14 : OAc decreased the relative catches of *Y. cag-*

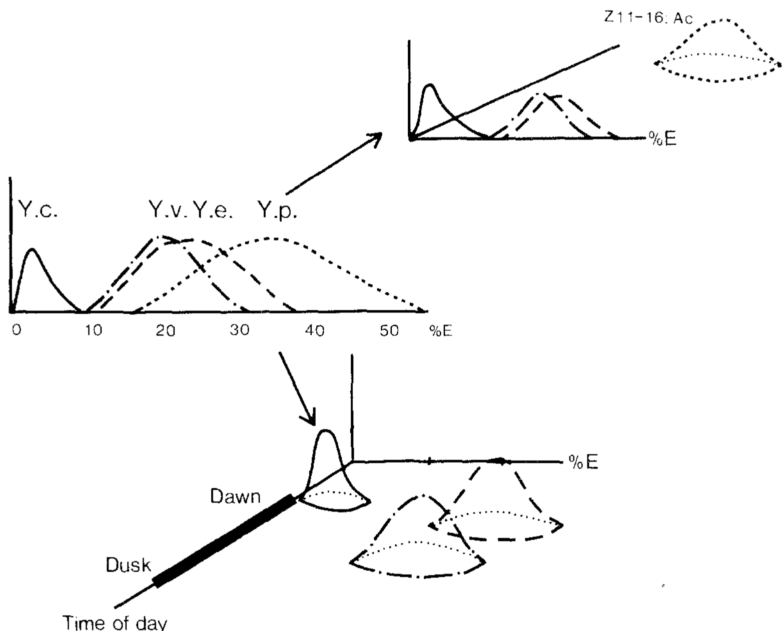


FIG. 5. Hypothetical graphical model of reproductive isolation in four European ermine moths, in three niche dimensions, i.e., the Z11- to *E*11-14 : OAc ratio, third components (Z11-16 : OAc) and time of the day when mating occurs (thick line = scotophase).

*nagellus.* Both these two pieces of information fit our model of reproductive isolation in *Yponomeuta.*

Within the limits of this study, the *Y. evonymellus* and *Y. vigintipunctatus* pheromones are similar. This is in accordance with the mutual interspecific attraction found by Ans Hendrikse (personal communication) in flight tunnel experiments and the mutual attraction in the field reported by Herrebout and van de Water (1983). The lack of chemical specificity is partly overcome by different diel periodicity of sexual activity and different seasonal occurrence. *Y. vigintipunctatus* is the only one of the four species presently discussed that is active around midnight (Hendrikse, 1979). *Y. vigintipunctatus* is also the only bivoltine European ermine moth (Herrebout et al., 1976). The first generation occurs in late May to early June and precedes all the other ermine moth flight periods. In southern Sweden the second generation starts to emerge in late July when the flight of *Y. evonymellus* already is declining (Löfstedt, unpublished). This does not rule out a possible contribution from different host-plant preferences if mating is restricted to the host plant (Heusinger, 1982; Herrebout and van de Water, 1982), different spatial niches (Herrebout and van de Water, 1982) or trace pheromone components as isolating factors, but such explanations do not seem necessary at present.

Morphological (Wiegand, 1962, as interpreted by Menken, 1962) as well as biochemical analysis (Menken, 1982) revealed that *Y. vigintipunctatus* is only distanctly related to *Y. evonymellus* and the five taxa referred to as the *padellus* complex. The divergence time between *Y. vigintipunctatus* and the rest of the species (Figure 1) was estimated by Menken (1982) to be approximately 12 million years, while the divergence time between *Y. evonymellus,* on one hand, and *Y. cagnagellus* and *padellus,* on the other, does not exceed one million years. Nevertheless the *Y. evonymellus* and *Y. vigintipunctatus* pheromones are very similar. Our interpretation of this is that the pheromone of a common *Yponomeuta* ancestor might have consisted of *Z*11-14:OAc, *E*11-14:OAc, and *Z*11-14:OH in proportions similar to those found in *Y. evonymellus* and *Y. vigintipunctatus.* Changes in the *Z/E* ratio and development of new biosynthetic pathways (e.g., a delta-11 desaturase working on the hexadecanoate) occurred as adaptive radiation.

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# SELECTIVE SEQUESTRATION OF MILKWEED (*Asclepias* sp.) CARDENOLIDES IN *Oncopeltus fasciatus* (DALLAS) (HEMIPTERA: LYGAEIDAE)

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**Abstract**—The cardenolide content of the gut, wings, and fat body of *Oncopeltus fasciatus* was examined. The female fat body contained 4–5% of the total cardenolide content of the insect. The cardenolide content of male fat body, and gut and wings of both sexes was below the detection limit of the cardenolide assay. Thin-layer chromatography was used to determine the cardenolide array of various tissues and secretions of *O. fasciatus* reared on seeds of a single species of milkweed (*A. speciosa*) and adult extracts and dorsolateral space fluid of *O. fasciatus* reared on seeds of two species of milkweed with different cardenolide arrays (*A. speciosa* and *A. syriaca*). Our results indicate that cardenolides are not sequestered in the insect simply on the basis of polarity and that metabolism and differential excretion of cardenolides are involved in the sequestration of cardenolides in *O. fasciatus*. The similarities in the cardenolide profiles of *O. fasciatus* reared on different food sources, and tissues of *O. fasciatus* reared on a single food source indicates that there is regulation of the cardenolide array in *O. fasciatus*.

**Key Words**—Cardenolide metabolism, cardenolide excretion, cardenolide sequestration, Hemiptera, Lygaeidae, *Oncopeltus fasciatus*, milkweed bug, *Asclepias*.

## INTRODUCTION

*Oncopeltus fasciatus* is a specialized feeder on plants containing cardenolides. Cardenolides from these host plants can be sequestered in the insect throughout its life cycle (Duffey and Scudder, 1974), and the insect suffers no apparent ill effects from the accumulation of these potentially toxic compounds (Isman, 1977; Chaplin and Chaplin, 1981). The cardenolides are stored primarily in the



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dorsolateral space, a highly vacuolated epithelial layer in the integument (Duffey and Scudder, 1974; Scudder and Meredith, 1982a). It has been suggested that differential distribution of cardenolides in the tissues of *O. fasciatus*, both in terms of quantity and polarity, function in the tolerance of the insect to large quantities of ingested cardenolides as well as in defense against predators (Duffey et al., 1978).

Blum (1981, 1983) has stressed the need for detailed studies of the specifics of sequestration of plant toxins by insects. The following study of the distribution of natural cardenolides in *O. fasciatus* was undertaken to clarify certain aspects of the differential distribution of natural cardenolides in *O. fasciatus*. The cardenolide content of three organs was examined: gut, wings, and fat body. These have not been investigated previously as possible sites of cardenolide accumulation. Retention of toxic compounds in the gut lumen or tissue and in the fat body may aid in the tolerance of toxins in insects (Kilby, 1963; Brooks, 1976), and in the monarch (*Danaus plexippus* L.) substantial amounts of plant cardenolides are found in both the gut lumen and the fat body (see Blum, 1981; 1983). Thus, it was of interest to see if cardenolides accumulate in the fat body and gut of *O. fasciatus*. In addition, large concentrations of cardenolides, thought to function in defense against predators, are sequestered in the wings of two lepidopterans, *Danaus plexippus* (Brower and Glazier, 1975; see Blum, 1981) and *Cycnia inopinatus* (Hy. Edwards) (see Blum, 1983) and in the elytra of the cerambycid beetle *Tetraopes melanurus* Schon. (Nishio et al., 1983). It is therefore possible that the wings of *O. fasciatus* also contain cardenolides.

To document some of the capabilities of the sequestration process of cardenolides in *O. fasciatus*, we determined, using thin-layer chromatography, the cardenolide array of various tissues and secretions of *O. fasciatus* reared on seeds of a single species of milkweed and of adult extracts and dorsolateral space fluid of *O. fasciatus* reared on seeds of two species of milkweed with very different cardenolide profiles. Thin-layer chromatography also allowed us to determine the polarity distribution of cardenolides in *O. fasciatus* and its tissues, which is of interest since the toxicity of cardenolides has been correlated with their polarity (Duffey, 1977; Smith and Haber, 1974; Detweiler, 1967). In addition, it has been suggested that the sequestration of cardenolides in *O. fasciatus* may be a function of their polarity (Duffey, 1980).

This study provides evidence that cardenolides are differentially distributed in *O. fasciatus* in terms of quantity, but not polarity, and that cardenolides are not sequestered in the insect simply on the basis of polarity. This study also provides evidence that metabolism and differential excretion of cardenolides are part of the selective sequestration process of cardenolides in *O. fasciatus*. In addition, the similarities in the cardenolide profiles of *O. fasciatus* reared on different food sources, and between tissues of *O. fasciatus* reared on a single food source, indicate that there is regulation of the cardenolide array in *O. fasciatus*.

## METHODS AND MATERIALS

*Insects.* Adult male and female and fifth instar larvae of *Oncopeltus fasciatus*, taken from a laboratory culture maintained at 26°C under a 16:8 light–dark cycle, were used in all experiments. The insects were reared either on commercial sunflower seeds (*Heliothus annuus* L.), or on milkweed seeds (*Asclepias speciosa* Torr. or *A. syriaca* L.). Insects reared on sunflower seeds provided controls since this food source does not contain cardenolides.

*Collection of Samples.* Gut (with contents) and fat body samples were dissected from insects in Berridge's dissecting saline initially (Berridge, 1966), and in later experiments in a saline based on the constituents of *O. fasciatus* hemolymph (Meredith et al., 1984). Tissues were rinsed in three 0.5-ml aliquots of saline and placed directly in extracting solvent or blotted 1 sec on each side and wet weight determined: no cardenolides were detected in the saline rinses of fat body or gut tissue. No attempt was made to remove tracheae or remnants of the membrane that encloses the fat body. Insects used to assay gut (with contents) were anesthetized for 30 sec with a low volume of $CO_2$ or cooled at 4°C. The gut was ligated with surgical thread (Ethicon, Inc.) at the Malpighian tubule–pylorus junctions, the anterior end of the 1st ventriculus and the posterior end of the hind gut, and removed by cutting distal to the ligatures: the ligatures ensured removal of the gut with its contents intact. Dissections were completed within 20 min.

Dorsolateral space fluid and hemolymph were collected from the insect as described by Duffey and Scudder (1974). Urine + feces samples were collected by placing insects for 48–72 hr in small rearing dishes lined with Whatman chromatography paper. An excess of seeds was provided and dechlorinated water was constantly available. After the collection period, the filter paper, which had absorbed the urine + feces, was removed and extracted for cardenolides. Filter paper from a control rearing dish ($N = 1$) set up identically to the rearing dishes for urine + feces samples but without insects was collected after 72 hr, extracted as for urine + feces samples, and assayed for cardenolides: none was detected. Wings were removed from insects after immobilization at 4°C.

*Extraction of Cardenolides.* With the exception of dorsolateral space fluid and hemolymph, cardenolides were extracted from all samples by either the insect or the seed method (Isman et al., 1977). The seed method was modified by retaining the $CHCl_3$ phase of the $CHCl_3$–MeOH 10:1 extraction for cardenolide determination. Lipids in some samples interfered with both TLC analysis and colorimetric determination of total cardenolide content. The seed method removed the interfering lipids and, therefore, was used (1) to extract all samples for TLC analysis (except urine + feces), and (2) with seed and fat body samples, used for total cardenolide determination. The insect method was used to extract whole insect, wing, and gut samples for total cardenolide determinations and urine + feces samples for TLC analysis.

All samples were extracted in a shaking incubator to facilitate diffusion of cardenolides and extraction liquors were concentrated to 10–20 $\mu l$ by evaporation under $N_2$ before application to TLC plates. Tests showed no difference in the cardenolide array in whole insect samples extracted by either method. For all samples, the petroleum ether discard of the seed method was assayed by TLC for cardenolides: none was detected. Even after seed method extraction, fat body extracts were difficult to apply to the TLC plate. Therefore, the extract was centrifuged (12,000*g*, 2 min) and the resulting two phases applied separately. Only one faint blue spot at the origin was detected in the lower phase. Dorsolateral space fluid and hemolymph were collected from the insect, precipitated with acetone (5:1 acetone–hemolymph) and 95% EtOH (1 $\mu l$ dorsolateral space fluid/ml 95% EtOH), centrifuged (12,000*g*, 2 min) to remove proteins, and the supernatant used for total cardenolide determination and TLC analysis of hemolymph. Dorsolateral space fluid was applied directly after collection to TLC plates. Resuspension of the pelleted hemolymph protein after 1 min of sonication in 95% EtOH yielded negligible cardenolide.

*Total Cardenolide Concentrations.* Total cardenolide concentrations were determined by a spectrophotometric assay using a Lambda 3 UV/VIS spectrophotometer (Perkin-Elmer) and the indicator 2,4,2′,4′-tetranitrodiphenyl (TNDP) in the presence of base (NaOH) (Brower and Moffitt, 1974; Brower et al., 1975). The TNDP reaction was run at room temperature. Sample absorbance at 626 nm was recorded 40 min after the colorimetric reaction was started, with 95% EtOH as the reference. Two controls were used to monitor any absorbance at 626 nm owing to substances in the individual sample extracts tested, as well as the reaction reagents TNDP and NaOH: control 1 = the absorbance of the particular insect sample being tested, in the absence of TNDP; control 2 = the absorbance of TNDP and NaOH, in the absence of insect sample. The absorbance of the two controls was subtracted from the absorbance of the experimental cuvette (insect sample, in the presence of TNDP and NaOH).

Cardenolide concentrations were determined by comparison to a digitoxin standard, and expressed as molar or microgram equivalent amounts of digitoxin ($\mu g_D$) to facilitate comparisons with other studies (Roeske et al., 1976). Brower and Glazier (1975), using the TNDP reaction, found only minor differences in the extinction coefficients of nine cardenolides and digitoxin. Therefore, it was assumed that digitoxin and the individual cardenolides ingested by the insects have similar extinction coefficients.

*Determination of Cardenolide Profiles with Thin-Layer Chromatography.* Thin-layer chromatography (TLC) plates prepared with silica gel G (Redi/Plate, Fisher Sci. Co., gel 250 $\mu m$ thick) were used. Plates were activated over concentrated $H_2SO_4$ for 24 hr prior to and 12 hr after applying samples (Duffey and Scudder, 1972). The relatively nonpolar cardenolide digitoxin was spotted as a standard on both sides of each sample. Plates were developed in filter-paper-

lined, saturated chambers containing methylene chloride–methanol–formamide (105 : 15 : 1) (Isman, personal communication) to a distance of 15–16 cm. TNDP followed by NaOH (Brower et al., 1982) were sprayed on the plates to detect cardenolides. To standardize results within and between TLC plates and obtain a measure of polarity of the cardenolides detected, TLC results were recorded as R digitoxin ($R_D$) values:

$$R_D = \frac{R_f \text{ spot}}{\overline{X}\, R_f \text{ of digitoxin on each side of sample}}$$

where $R_f$ = distance spot moved from origin/distance solvent front moved from origin. The $R_f$ of digitoxin was 0.51 ± 0.003 ($N$ = 18).

The cardenolides of *A. speciosa* and *A. syriaca* seeds have not been identified. In addition, since $R_D$ values vary between different samples of a given tissue and between TLC plates, we used the following characteristics to identify individual cardenolides: (1) color (red or blue, red indicating a noncardenolide); (2) $R_D$ value; (3) pattern (based on color, relative position to other cardenolides in sample, and intensity). Intensity is an indication of concentration (Brower et al., 1982). The detection limit of the TLC assay was 0.3–0.5 μg of digitoxin. For a further indication of polarity, the $R_D$ values of the very polar cardenolide ouabain and the nonpolar cardenolide digitoxigenin were determined and found to be 0.081 ($N$ = 3), and 1.22 ± 0.010 ($N$ = 9), respectively. Although samples from males and females were assayed separately, no sexual differences were found (except where noted in Figure 1) and results from both sexes were pooled. The cardenolide profiles were compiled from TLC analysis of 4–16 different extracts of each tissue or secretion. To ensure detection of cardenolides present in low concentrations, the amount of insect material used for the different extracts of the individual tissues was varied.

The following control samples from insects reared on *H. annuus* were assayed for cardenolide profiles: adult whole insects [1 male (M), 1 female (F)], urine + feces (10 F, 10 M excreting for 72 hr), eggs (100 and 200), hemolymph (2 replicates each of ½ extract of 33 M and 25 F), and dorsolateral space fluid (3 F and 3 M). No cardenolides were detected in any of the samples.

## RESULTS

*Total Cardenolide Content of Fat Body, Wings, Gut, and Dorsolateral Space Fluid of* O. fasciatus *Reared on* A. speciosa. Our results indicate that large amounts of cardenolides do not accumulate in the fat body, wings, or gut of adult *O. fasciatus* reared on *A. speciosa* (Table 1). The concentration of cardenolides in gut and wing extracts of both sexes and fat body samples of adult males was below the detection limit of the assay (10 $\mu g_D$). Fat bodies of

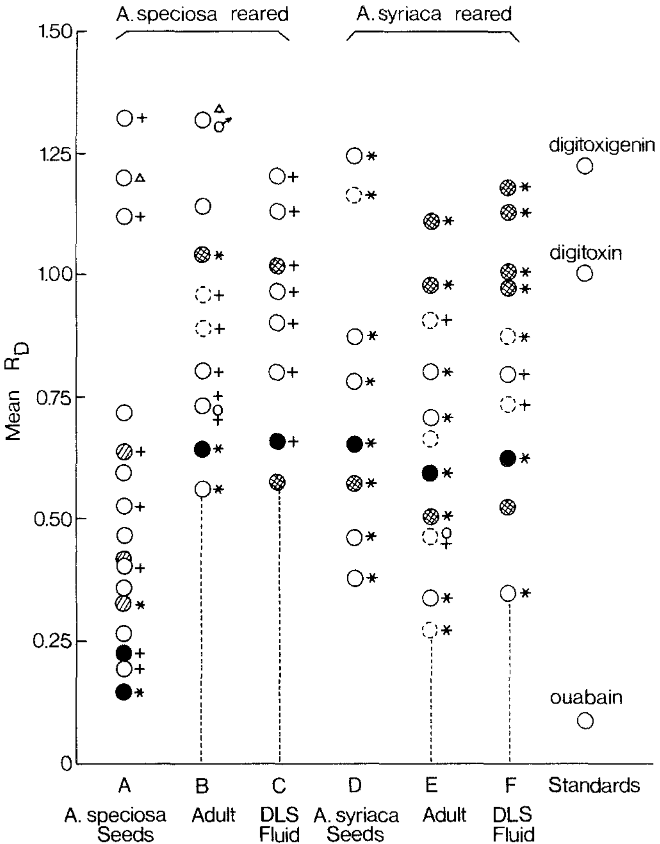


FIG. 1. Cardenolide profiles of *A. speciosa* and *A. syriaca* seeds and adult extracts and dorsolateral space (DLS) fluid of *O. fasciatus* reared on each seed. The relative concentrations of the cardenolides of the different samples are not comparable. Symbols: ◍, red (noncardenolide); ◌, faint blue; ○, blue; ⊕, dark blue; ●, very dark blue; ◍, dark blue or very dark blue depending on the extract; △, seen in only one extract; +, seen in >50% of extracts; *, seen in all samples; ♂, seen in male only; ♀, seen in female only; ⁞, light blue tailing. Sample sizes: *A. speciosa* seeds and samples of insects reared on *A. speciosa* as described in Figure 2. *A. syriaca* seeds, $N = 4$; adults, $N = 8$ (4 ♂, 4 ♀); dorsolateral space fluid, $N = 8$ (4 ♂, 4 ♀), fluid from 1-3 adults per sample.

adult females contained only 4–5% (mean 8 $\mu g_D$) of the total cardenolide content detected in whole females. Similarly, the fat body of male and female fifth instar larvae does not appear to be a major site of cardenolide accumulation. Using half the total cardenolide content of adults as a conservative estimate for the concentration of cardenolides in fifth instar larvae (Duffey and Scudder, 1974), the cardenolides sequestered in the fat body of such larvae account for less than 12% of the total cardenolides stored at this stage.

TABLE 1. TOTAL CARDENOLIDE CONTENT OF ADULT AND 5th INSTAR *Oncopeltus fasciatus* SAMPLES[a]

| Sample | No. in each sample | Total no. of samples | Cardenolide: $\mu g_D$/insect or organ | Cardenolide: $\mu g_D$/mg wet weight insect or organ |
|---|---|---|---|---|
| Whole insect (adult) | | | | |
| Female[b] | 1 | 10 | 208.3 ± 23.6 (100.8–336.1) | 4.9 ± 0.4 (2.9–7.5) |
| Male[b] | 1 | 10 | 208.8 ± 21.1 (99.6–293.8) | 5.8 ± 0.6 (2.2–7.9) |
| Female[c] | 1 | 8 | 152.5 ± 9.6 (103.4–196.1) | 3.4 ± 0.3 (1.6–5.9) |
| Male[c] | 1 | 8 | 148.0 ± 4.3 (66.8–191.6) | 4.5 ± 0.2 (1.3–7.8) |
| Fat body[d] | | | | |
| Female adult | 6–7 | 3 | 8.0 (4.0–10.6)[e] | 4.0 ± 1.0 (2.0–5.3) |
| Female 5th instar | 6–7 | 2 | 8.4 (8.0–9.0)[e] | 2.5 ± 0.1 (2.4–2.7) |
| Male adult | 6 | 3 | BDL[d] | BDL |
| Male 5th instar | 7 | 2 | 5.3 (4.9–5.6)[e] | 1.4 ± 0.1 (1.3–1.5) |
| Gut with contents (adult)[d] | | | | |
| Female | 4 | 2 | BDL | BDL |
| Male | 4 | 2 | BDL | BDL |
| Wings (set = fore + hind)[c] | | | | |
| Female | 1 set | 4 | BDL | BDL |
| Male | 1 set | 4 | BDL | BDL |
| Dorsolateral space fluid (adult)[c] | | | | |
| Female | 1 $\mu$l | 9 | 69.8–104.7[f] | – |
| Male | 1 $\mu$l | 11 | 88.0–132.0[f] | – |

[a]Cardenolide content is measured in digitoxin equivalents ($\mu g_D$) and is reported as mean ± SE (range). BDL = below detection limits of assay. All insects were reared on *Asclepias speciosa* seeds collected in Penticton, B.C.
[b]Reared on seeds collected September 1981.
[c]Reared on seeds collected September 1982.
[d]Reared on seeds collected September 1980.
[e]Calculated by multiplying $\mu$g digitoxin equivalent per mg wet weight by mean fat body mass/insect.
[f]Calculated by multiplying $\mu$g digitoxin equivalent per $\mu$l dorsolateral space fluid by estimated volume of dorsolateral space fluid/insect (2–3 $\mu$l; Duffey and Scudder, 1974). Mean determined concentration of cardenolides in dorsolateral space fluid for adult females and males is 34.9 ± 4.4 (range 24.2–67.0) and 44.0 ± 3.9 (range 17.0–58.3) $\mu g_D/\mu l$ fluid, respectively.

In both sexes, cardenolide accumulation in the fat body appears to change during development from fifth instar larvae to adult (Table 1). The fat body of female fifth instar larvae and adults contained similar total amounts of cardenolides (mean 8.4, range 8.0–9.0 and mean 8.0, range 4.0–10.6, $\mu g_D$/fat body, respectively). However, when expressed as $\mu g_D$ per mg wet weight, on average $\frac{1}{3}$ more cardenolides were detected in the adult (mean 4.0, range 2.0–5.3, vs. mean 2.5, range 2.4–2.7, $\mu g_D$/mg fat body). Measureable amounts of cardenolides were detected only in the fat body of male fifth instar larvae; none was detected in the adult. In both the fifth instar and adult, the male fat body contained lower concentrations of cardenolides than the female.

The cardenolide content of the fat body of adult females may be affected by reproductive state. The cardenolide content of three samples of six to seven fat bodies was determined; the average cardenolide content of the fat bodies in two of the samples was almost twice that found in the third sample (9.1 and 8.2 vs. 4.8 $\mu g_D$/fat body). In the third sample, half the insects used had well developed ovarioles and eggs, whereas none of the individuals in the other two samples contained eggs in the ovarioles and were probably young females prior to egg development.

The greatest concentration of cardenolides was found in the dorsolateral space fluid, with a mean of 34.9 $\pm$ 4.4 (range 24.2–67.0) $\mu g_D/\mu l$ fluid in females and a mean of 44.0 $\pm$ 3.9 $\mu g_D/\mu l$ (range 17.0–58.3) fluid in males. The difference between the cardenolide content of dorsolateral space fluid in males and females is not significant (Scheffe's test for multiple comparisons). Using 2–3 $\mu l$ as an estimated total volume of dorsolateral space fluid in adult *O. fasciatus* (Duffey and Scudder, 1974), the cardenolides in the dorsolateral space fluid account for 46–89% of the cardenolides in the insect. The sequestration sites of the cardenolides unaccounted for in this study of cardenolide distribution in tissues of *O. fasciatus* are unknown. It is possible that the cardenolides in the dorsolateral space fluid account for a greater percentage of the cardenolides in the insect than we estimated, since it is difficult to determine the total vacuolar volume of the inner epithelial layer of the epidermis.

The total cardenolide content of adult *O. fasciatus* was determined for insects reared on *A. speciosa* collected from Penticton, British Columbia, in September 1981 and September 1982. Less cardenolides were sequestered by insects reared on seeds collected in 1982 [mean 150 (range 67–196) $\mu g_D$ vs. mean 208 (range 100–336) $\mu g_D$/insect]. The cardenolide content of males and females did not differ significantly on a per insect basis when reared on seeds collected either year or on a wet weight basis for insects reared on September 1981 seeds (Student's $t$ test). However, the cardenolide content per milligram wet weight of males was significantly greater than females for insects reared on September 1982 seeds (4.5 vs. 3.4 $\mu g_D$/mg wet weight, $P < 0.01$, Student's $t$ test).

*Cardenolide Profiles of* O. fasciatus *Reared on* A. speciosa *and* A. syriaca *Seeds.* To investigate the potential capabilities and limitations of cardenolide

sequestration in *O. fasciatus*, the differences and similarities in the cardenolide array of dorsolateral space fluid and adult extracts from insects reared on the seeds of two different species of milkweed (*A. speciosa* collected in Penticton, British Columbia, and *A. syriaca* collected in Willimantic, Connecticut) were determined by TLC analysis. Since only minor differences were detected between males and females (♂, ♀, Figure 1), data from both sexes were pooled. Table 2 lists the $R_D$ values and identities of the cardenolides in each profile. The cardenolides of *A. speciosa* and *A. syriaca* seeds have not been identified; therefore, cardenolides were identified in this study by color, $R_D$ value, and pattern (based on color, relative position to other cardenolides in sample, and intensity).

Cardenolides of a wide polarity range were available from *A. speciosa* seeds ($R_D$ 0.14–1.31; A, Figure 1). However, insects reared on this food source preferentially sequestered cardenolides of a more limited polarity range: very nonpolar ($R_D > 1.20$) and polar cardenolides ($R_D < 0.50$) were absent or in low concentrations (B, C, Figure 1, Table 2). A nonpolar cardenolide ($R_D$ 1.32) was detected in male *O. fasciatus* and polar cardenolides were present in the fat body, but these were in very low concentrations (see following section). A more limited polarity range of cardenolides was present in *A. syriaca* seeds ($R_D$ 0.38–1.24; D, Figure 1). Very nonpolar cardenolides were not detected in insects reared on this food source either. However, polar cardenolides ($R_D < 0.50$) were sequestered in greater concentrations in insects reared on *A. syriaca* than in those reared on *A. speciosa* (E, F, Figure 1).

The cardenolide of highest concentration in insects reared on either seed was of intermediate polarity ($R_D$ approx. 0.62; ●, Figure 1; spot 19, Table 2). Another cardenolide of intermediate polarity ($R_D$ approx. 0.57; spot 16, Table 2; ⊗, Figure 1) and less polar cardenolides in the same polarity range as digitoxin ($R_D$ 1.0; ⊗, Figure 1) were concentrated in insects reared on either species of milkweed seed. In addition, fairly nonpolar cardenolides ($R_D$ 1.12 and 1.17; spots 32 and 33, Table 2) were concentrated in insects reared on *A. syriaca*. Cardenolides of these $R_D$ values were present in *A. speciosa*-reared insects, but were in lower concentrations.

In *A. syriaca*-reared insects, four cardenolides in the $R_D$ range of 1.0–1.2 were concentrated in the dorsolateral space fluid, but only two diffuse spots were detected in this range in whole adult extracts. It is possible that all four cardenolides were present in the adult extracts, but could not be separated owing to interference from waxes, lipids, or other compounds in the adult extracts.

When reared on either *A. syriaca* or *A. speciosa* seeds, *O. fasciatus* sequestered most of the cardenolides present in the seeds. Nine of the 13 cardenolides detected in extracts of *A. speciosa* seeds and six of the eight cardenolides detected in extracts of *A. syriaca* seeds corresponded to cardenolides in insects reared on these seeds (Figures 1 and 2, Table 2). However, 12 cardenolides detected in insects reared on *A. speciosa* did not correspond to *A. speciosa* cardenolides, 10 of these occurring in $R_D$ ranges where no cardenolides were

TABLE 2. $R_D$ VALUES OF CARDENOLIDES AND NONCARDENOLIDES (NC)[a] DETECTED IN EXTRACTS OF *O. fasciatus* AND ITS FOOD SOURCES, *A. speciosa* OR *A. syriaca*[b]

| | Extracts from insects reared on *A. speciosa* seeds | | | | |
|---|---|---|---|---|---|
| TLC spot number | *A. speciosa* seeds (9) | *O. fasciatus* adults (10) | Dorsolateral space fluid (15) | hemolymph (4) | eggs (7) |
| 1 | – | – | – | – | – |
| 2 | – | – | – | – | – |
| 3 | 0.14 ± 0.005 | – | – | – | – |
| 4 NC | – | – | – | – | – |
| 5 | 0.19 ± 0.002 | – | – | – | – |
| 6 | 0.22 ± 0.003 | – | – | – | – |
| 7 NC | – | – | – | – | – |
| 8 | 0.26 ± 0.004 | – | – | – | – |
| 9 | – | – | – | – | – |
| 10 NC | 0.32 ± 0.008 | – | – | – | – |
| 11 | 0.35 ± 0.002 | – | – | – | – |
| 12 | 0.40 ± 0.009 | – | – | – | – |
| 13 NC | 0.41 ± 0.007 | – | – | – | – |
| 14 | 0.46 ± 0.006 | – | – | – | – |
| 15 | 0.52 ± 0.012 | – | – | – | – |
| 16 | – | 0.56 ± 0.008 | 0.58 ± 0.007 | 0.56 ± 0.009 | 0.55 ± 0.008 |
| 17 | 0.59 ± 0.016 | – | – | – | – |
| 18 NC | – | – | – | – | – |
| 19 | – | 0.64 ± 0.007 | 0.66 ± 0.008 | 0.66 ± 0.009 | 0.63 ± 0.008 |
| 20 NC | 0.63 ± 0.011 | – | – | – | – |
| 21 | – | – | – | – | – |
| 22 NC | – | – | – | – | – |
| 23 | 0.71 ± 0.006 | – | – | – | 0.71 |
| 24 | – | 0.73 ± 0.009 | – | – | 0.76 ± .009 |
| 25 | – | 0.80 ± 0.009 | 0.80 ± 0.008 | 0.79 ± 0.007 | 0.81 |
| 26 | – | – | – | 0.86 ± 0.010 | 0.86 ± 0.002 |
| 27 | – | 0.89 ± 0.009 | 0.90 ± 0.006 | – | 0.88 |
| 28 | – | – | – | 0.94 ± 0.010 | 0.94 |
| 29 | – | 0.96 ± 0.005 | 0.97 ± 0.003 | – | 0.96 ± 0.006 |
| 30 | – | 1.04 ± 0.012 | 1.02 ± 0.005 | 1.00 ± 0.006 | 1.02 ± 0.010 |
| 31 | – | – | – | – | 1.08 ± 0.003 |
| 32 | 1.10 ± 0.009 | 1.14 ± 0.010 | 1.13 ± 0.009 | 1.11 ± 0.004 | – |
| 33 | 1.19 | – | 1.20 ± 0.009 | – | – |
| 34 | – | – | – | – | – |
| 35 | 1.31 ± 0.014 | 1.32 | – | – | – |
| Total number of cardenolides | 13 | 9 | 8 | 7 | 11 |

[a]Noncardenolides (NC) are compounds that turned red when sprayed with colorimetric reagents, cardenolides color blue or purple.
[b]($N$) = number of samples. $R_D$ values are reported as mean ± SE.

TABLE 2. CONTINUED.

| TLC spot number | Extracts from insects reared on *A. speciosa* seeds: Fat body (females) (7) | Urine/feces (16) | *A. syriaca* seeds (4) | Extracts from insects reared on *A. syriaca* seeds: *O. fasciatus* adults (7) | Dorsolateral space fluid (8) |
|---|---|---|---|---|---|
| 1 | 0.00 ± 0.000 | – | – | – | – |
| 2 | 0.05 ± 0.003 | – | – | – | – |
| 3 | – | – | – | – | – |
| 4 NC | 0.14 ± 0.004 | – | – | – | – |
| 5 | 0.19 ± 0.005 | 0.18 ± 0.003 | – | – | – |
| 6 | – | – | – | – | – |
| 7 NC | 0.23 ± 0.009 | – | – | – | – |
| 8 | – | – | – | – | – |
| 9 | 0.31 ± 0.005 | – | – | 0.27 ± 0.006 | – |
| 10 NC | – | – | – | – | – |
| 11 | 0.36 ± 0.005 | – | – | – | – |
| 12 | 0.41 | 0.42 ± 0.003 | 0.38 ± 0.007 | 0.34 ± 0.004 | 0.34 ± 0.005 |
| 13 NC | – | – | – | – | – |
| 14 | 0.48 | – | 0.46 ± 0.005 | 0.46 | – |
| 15 | – | 0.52 ± 0.007 | – | – | – |
| 16 | – | 0.56 ± 0.005 | 0.57 ± 0.007 | 0.50 ± 0.004 | 0.52 ± 0.004 |
| 17 | – | – | – | – | – |
| 18 NC | – | 0.60 ± 0.010 | – | – | – |
| 19 | 0.64 ± 0.003 | 0.61 ± 0.006 | 0.65 ± 0.007 | 0.59 ± 0.005 | 0.62 ± 0.012 |
| 20 NC | – | – | – | – | – |
| 21 | – | – | – | 0.66 | – |
| 22 NC | – | 0.67 | – | – | – |
| 23 | – | – | – | 0.70 ± 0.008 | – |
| 24 | – | 0.75 ± 0.015 | – | – | 0.73 ± 0.008 |
| 25 | 0.78 | – | 0.78 ± 0.003 | 0.79 ± 0.004 | 0.79 ± 0.007 |
| 26 | – | 0.86 ± 0.008 | – | – | – |
| 27 | – | – | 0.87 ± 0.003 | 0.90 ± 0.003 | 0.87 ± 0.009 |
| 28 | – | – | – | – | – |
| 29 | 0.96 | 0.95 ± 0.009 | – | 0.97 ± 0.009 | 0.97 ± 0.007 |
| 30 | 1.02 | 1.01 ± 0.018 | – | – | 1.02 ± 0.007 |
| 31 | – | – | – | – | – |
| 32 | – | 1.12 ± 0.004 | – | 1.10 ± 0.007 | 1.12 ± 0.008 |
| 33 | – | – | 1.16 ± 0.006 | – | 1.17 ± 0.007 |
| 34 | – | – | 1.24 ± 0.007 | – | – |
| 35 | – | – | – | – | – |
| Total number of cardenolides | 13 | 12 | 8 | 11 | 10 |

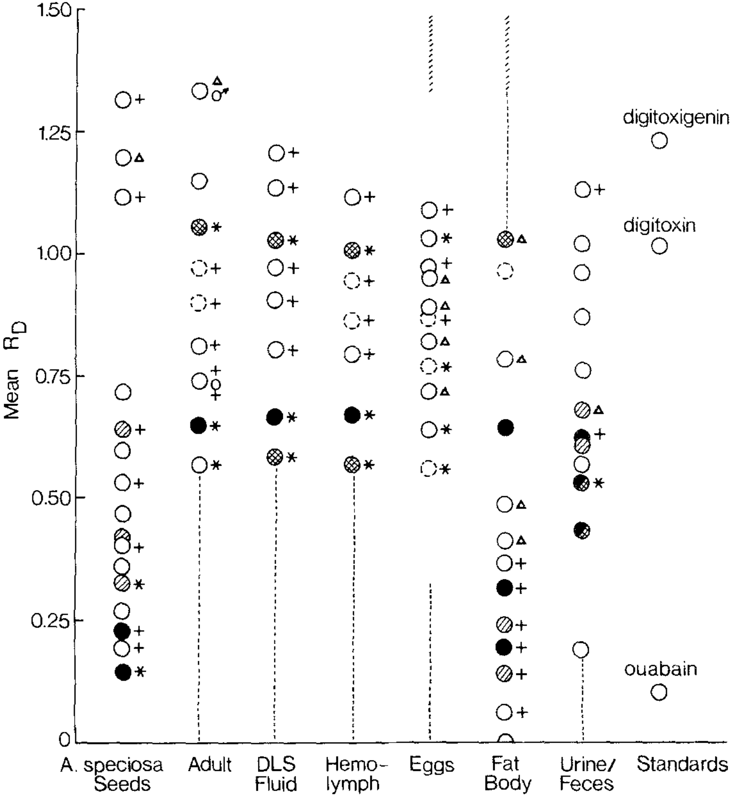


FIG. 2. Cardenolide profiles of *O. fasciatus* adults, tissues, and secretions and *A. speciosa* seeds. The relative concentrations of the cardenolides of the different samples are not comparable. Symbols as described in Figure 1; ⦚, red tailing. Sample sizes: *A. speciosa* seeds, $N = 9$, 0–5 replicates per sample; adults, $N = 10$ (7 ♀, 3 ♂), 1–5 adults and 0–1 replicate per sample; dorsolateral space (DLS) fluid, $N = 15$ (7 ♀, 8 ♂), fluid from 1–3 individuals and no replicates per sample. Hemolymph, $N = 4$ (2 ♀, 2 ♂), hemolymph from 24–30 individuals and three replicates per sample; eggs, $N = 7$, 25–200 eggs and no replicates per sample; fat body, $N = 7$ (all females), 10–15 fat bodies and 0–1 replicate per sample; urine + feces, $N = 16$ (7 ♀, 9 ♂), 180–1920 excrement hours (EH) = No. of insects excreting × No. hours insects excreted, 0–4 replicates per sample.

detected in the seeds ($R_D$ 0.00–0.05 and 0.75–1.05, Figures 1 and 2, Table 2). Similarly, eight of the cardenolides found in insects reared on *A. syriaca* did not correspond to *A. syriaca* seed cardenolides (Figure 1, Table 2). Four of these insect cardenolides occurred in $R_D$ ranges where no cardenolides were detected in *A. syriaca* seeds ($R_D$ 0.27 and 0.97–1.12). The presence of cardenolides in *O. fasciatus* that were not detected in its food source indicates metabolism of

seed cardenolides in the seeds by salivary enzymes of *O. fasciatus*, metabolism within the insect, or concentration in the insect of seed cardenolides that were in such low levels in the seeds they were not detected in TLC analysis.

The cardenolide profiles of adult extracts and dorsolateral space fluid of insects reared on the two different milkweed seeds were quite similar. Nine of the 10 cardenolides detected in adults and dorsolateral space fluid of insects reared on *A. speciosa* seeds corresponded closely to cardenolides found in the same extracts of *A. syriaca* reared insects (Table 2). Only four of the 13 cardenolides present in adult extracts and dorsolateral space fluid of *O. fasciatus* reared on *A. syriaca* were not found in the same extracts of *A. speciosa*-reared insects. However, they were detected in low concentrations in extracts of other tissues of *A. speciosa* reared insects (spots 9, 12, 14, 24; Table 2). In addition, the highly concentrated cardenolides in insects reared on *A. speciosa* (●, ❁, Figure 1; spots 16, 19, 30, Table 2) corresponded to three of the most concentrated cardenolides in insects reared on *A. syriaca*.

The similarities in the cardenolide profiles of *O. fasciatus* reared on the two different milkweed seeds do not reflect similarities in the cardenolide array of the two seeds. Only two of the nine cardenolides found in common in adult extracts and dorsolateral space fluid of *O. fasciatus* reared on either food source were found in both species of milkweed seeds. Furthermore, two of the highly concentrated cardenolides in *A. syriaca*-reared insects were also the most concentrated cardenolides in their food source (spots 16 and 19, Table 2), whereas these same cardenolides, although highly concentrated in *A. speciosa*-reared insects, did not correspond to cardenolides in *A. speciosa* seeds.

*Cardenolide Profiles of Adult Extracts, Tissues, and Dorsolateral Space Fluid Reared on* A. speciosa *Seeds.* To determine if different cardenolides were preferentially sequestered in different tissues of *O. fasciatus*, the cardenolide array of adult extracts, dorsolateral space fluid, urine + feces, fat body, hemolymph, and egg extracts of insects reared on *A. speciosa* seeds was investigated by TLC analysis.

The cardenolide profiles of all the extracts tested were similar in terms of the individual cardenolides sequestered and their relative concentrations (Figure 2, Table 2). The cardenolide array of both sexes was analyzed for each extract, but, since only minor differences were detected between male and female samples (♂, ♀, Fig. 2), the data from both sexes were pooled. The cardenolide arrays of the adult, dorsolateral space fluid, hemolymph, and eggs showed the greatest similarity: $\frac{7}{8}$, $\frac{5}{7}$ and $\frac{7}{11}$ (87, 71, and 64%) of the cardenolides detected in the dorsolateral space fluid, hemolymph, and eggs, respectively, corresponded to the adult cardenolides. The most concentrated cardenolide in the insect ($R_D$ 0.64) was also the most concentrated cardenolide in the dorsolateral space fluid, hemolymph, and eggs. This cardenolide was also concentrated in the fat body and urine + feces, but both extracts contained two more polar cardenolides of similar concentration.

The cardenolide arrays of the fat body and urine + feces were similar to the other insect tissues in the intermediate polarity range $R_D$ 0.55–1.10, but differed owing to the presence of low concentrations of cardenolides in the polar range $R_D < 0.50$ and the concentration of both intermediate and more polar cardenolides (●, ⊗, Figure 2). Large amounts of urine + feces, fat body, and eggs were needed to obtain extracts with cardenolide concentrations high enough to visualize in TLC analysis. As a consequence, the detection of cardenolides in low concentrations was enhanced in these tissues and may explain, in part, the greater number of cardenolides detected in these samples and the detection of cardenolides in the polar range $R_D < 0.50$ in fat body and urine + feces samples. Large sample sizes could not be used for the other insect samples because of severe tailing of highly concentrated cardenolides. Cardenolides in the polar range $R_D < 0.50$ may be present in low concentrations in the other samples since faint blue tailing was detected between the origin and $R_D$ 0.55 in most samples (¦, Figure 2). Alternatively, this tailing may represent nonspecific binding of cardenolides to various compounds such as proteins, waxes, pigments, or lipids.

Very nonpolar cardenolides were absent or in low concentrations in all extracts of the insect. Adult and dorsolateral space fluid extracts contained cardenolides of the lowest polarity ($R_D$ 1.32 and 1.20, respectively), which correspond to the most nonpolar cardenolides detected in the seeds. However, the very nonpolar cardenolide ($R_D$ 1.32) in the adult male was apparently in very low concentrations: it was only detected in a pooled sample of five males, never in extracts of single males. Low concentrations of very nonpolar cardenolides may also occur in the fat body since extracts produced faint blue tailing from $R_D$ 1.04 to approximately 1.32 (¦, Figure 2).

With the exception of urine + feces, the array of cardenolides detected in different extracts of the same insect tissue was quite constant (Table 3; + and * in Figure 1). Much of the variability between extracts of the same tissue was the result of the difficulty in detecting cardenolides of low concentrations; owing to their weak intensity and/or masking by tailing of cardenolides of higher concentrations, they were difficult to detect. However, most of the cardenolides detected in urine + feces occurred in less than half the extracts tested, indicating a real difference in cardenolide profiles between samples. In addition, the cardenolides of greatest concentration varied between samples of urine + feces (⊗, Figure 2), whereas in different extracts of all other tissues, the cardenolide of greatest concentration was constant (●, Figures 1 and 2).

*Geographic Differences in Cardenolide Profile of* A. syriaca *Seeds.* Previous studies of sequestration of asclepiad cardenolides in *O. fasciatus* used *A. syriaca* seeds from Missouri and Ontario as the insects' food source (Feir and Suen, 1971; Duffey and Scudder, 1974). It is possible that differences in the results between these studies and the present study are a result of geographic

TABLE 3. VARIABILITY IN CARDENOLIDE PROFILES OF *O. fasciatus* AND *A. speciosa*[a]

| Sample | Percent |
|---|---|
| *A. speciosa* seeds | 56 |
| Urine + feces | 25 |
| Adults | 70 |
| Eggs | 64 |
| Hemolymph | 100 |
| Dorsolateral space fluid | 100 |
| Fat body | 62 |

[a]Variability is expressed as the percentage of individual cardenolides seen in 50% or more of the extracts of each sample.

variation in the cardenolide profile of seeds of *A. syriaca*. Geographical variation has not been established for the seeds of *A. syriaca;* therefore, we investigated this by determining the cardenolide array of *A. syriaca* seeds from the following four locations: Montebello, Quebec (collected October, 1976); Ottawa, Ontario (collected September, 1976); Cleveland, Ohio (collected September, 1975); and Willimantic, Connecticut (collected September 1981). The cardenolide array of the seeds collected in Ontario, Ohio, and Quebec showed only minor differences in the individual cardenolides present and their relative concentrations, but were dissimilar to the cardenolide profile of the Connecticut seeds (Figure 3).

## DISCUSSION

This study was undertaken to clarify certain aspects of the differential distribution of natural cardenolides in *O. fasciatus*, both in terms of quantity and polarity, and to document some of the capabilities of the sequestration process of cardenolides in *O. fasciatus*. Our results show that large amounts of cardenolides to not accumulate in the fat body, gut, and wings of *O. fasciatus;* the greatest amount of cardenolides in the insect is in the dorsolateral space. The distribution of cardenolides in *O. fasciatus* is summarized in Table 4.

Blum (1983) has suggested that each species of insect has a unique process of sequestration, tolerance, and defensive use of the toxins ingested with its food plant. The differences in the distribution of cardenolides in *O. fasciatus* and two other insects, *Danaus plexippus* and *Cycnia inopinatus*, support this argument. Low amounts of cardenolides are sequestered in the wings, fat body, gut, and hemolymph of *O. fasciatus*, whereas substantial amounts of cardenolides are found in these tissues in the monarch (see Blum, 1981, 1983). *C. inopinatus* differs from both the monarch and *O. fasciatus* in the presence of large amounts

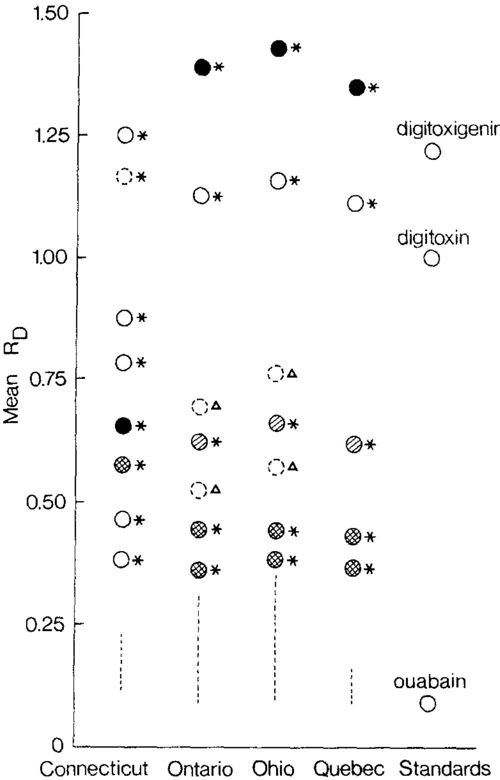


FIG. 3. Cardenolide profiles of *A. syriaca* seeds from four geographic locations. Symbols as described in Figure 1. Sample sizes: $N = 2$ for Ontario, Ohio, and Quebec seeds; $N = 4$ for Connecticut seeds.

of cardenolides in the hemolymph and wings, but negligible amounts of these compounds in the gut (see Blum, 1983).

It appears that *O. fasciatus*, unlike *D. plexippus* and *C. inopinatus*, may not tolerate large quantities of cardenolides in the hemolymph. Thus, the rapid uptake and accumulation of cardenolides in the dorsolateral space may function in the insect's tolerance of sequestered cardenolides by maintaining low levels of cardenolides in the hemolymph (Duffey et al., 1978) and other tissues, i.e., fat body and wings (this study). Furthermore, the low cardenolide content of the wings in *O. fasciatus* indicates that the antipredator strategies of *O. fasciatus* may differ from *D. plexippus* and *C. inopinatus*. In the monarch, the greatest concentration of cardenolides is in the wings, and this is thought to function as an antipredator strategy, causing avian predators to reject the butterfly relatively unharmed and preventing attack of the more critical areas: the thorax and abdomen (Brower and Glazier, 1975). It is unlikely that the wings of *O. fasciatus*

TABLE 4. DISTRIBUTION OF CARDENOLIDES IN *Oncopeltus fasciatus* EXPRESSED AS PERCENT OF ADULT TOTAL

| Sample | % of Adult total | Reference[a] |
|---|---|---|
| Dorsolateral space | 60–95 | 1 |
| Dorsolateral space fluid | 46–89 | 2[c] |
| Hemolymph | BDL[b] | 1,2[d] |
| Urine + feces | BDL | 1 |
| Metathoracic gland | BDL | 1 |
| Fat body (male) | BDL | 2 |
| Fat body (female) | 4–5 | 2 |
| Gut with contents | BDL | 2 |
| Wings | BDL | 2 |

[a]1 = Duffey and Scudder, (1974) using *Asclepias syriaca* for the food source. 2 = This study, using *A. speciosa* for the food source.
[b]Below detection limit of assay.
[c]Calculated using estimate of 2–3 μl for volume of dorsolateral space fluid in adult (Duffey and Scudder, 1974).
[d]Unpublished results.

would function effectively in this manner. Owing to the small body size of *O. fasciatus* (17–18 mm) and the positioning of the wings at rest flat along the back, large predators probably snatch the entire insect upon attack. Thus, for *O. fasciatus*, concentration of cardenolides in the dorsolateral space and release of dorsolateral space fluid along the thorax and abdomen is probably a more effective antipredator strategy (Duffey and Scudder, 1974; Scudder and Meredith, 1982a).

The results from the TLC analysis indicate that there are only minor differences in the cardenolides sequestered and concentrated in various tissues and secretions of *O. fasciatus* reared on seeds of a single species of milkweed. The presence in the fat body of low concentrations of polar cardenolides not detected in other tissues may indicate a difference in the cardenolide array sequestered in this tissue. Alternatively, this difference may be the result of enhanced detection of cardenolides in low concentrations in the fat body (see Results).

Only minor differences were detected in the cardenolide profiles of adult extracts and dorsolateral space fluid of *O. fasciatus* reared on two food sources with very different cardenolide arrays. This indicates that the cardenolide array of the insect can remain fairly constant despite differences in the cardenolide profiles of its food plants. These results differ from studies in which variation in cardenolide profiles of monarchs has been related to interspecific differences in the cardenolide array of their food plants (Roeske et al., 1976; Brower et al., 1984). However, two other studies of sequestration of cardenolides in *O. fasciatus* reared on *A. syriaca* seeds (Feir and Suen, 1971; Duffey and Scudder, 1974) suggest that the cardenolide profile of *O. fasciatus* may exhibit differences

related to intraspecific variation in the cardenolide profiles of its food plant. In our study, seven cardenolides were detected in insects reared on *A. syriaca* seeds collected in Connecticut, and most of the cardenolides in the seeds were sequestered in the insect. In contrast, Feir and Suen (1971) found only four cardenolides were sequestered in insects reared on *A. syriaca* seeds collected in Missouri, and only one of these corresponded to a seed cardenolide. We found only minor differences in the cardenolide profiles of adult extracts and dorsolateral space fluid of insects reared on *A. syriaca* seeds from Connecticut, and the highly concentrated cardenolides in the insect covered a wide range of polarities. However, insects reared on *A. syriaca* seeds from Ontario exhibited a predominance of polar cardenolides, and the cardenolides of the dorsolateral space fluid exhibited a much smaller polarity range than seen in the whole insect (Duffey and Scudder, 1974).

The differences in the cardenolide profiles of *O. fasciatus* in these studies may be explained, in part, by the different extraction methods, solvent systems, and detection reagents used in the TLC analyses. In addition, the differences in the cardenolide arrays of the insect may reflect variation in the cardenolide profiles of the *A. syriaca* seeds the insects were reared on. Geographic variation in the cardenolide array of vegetative parts of *A. syriaca* has been well established (see Roeske et al., 1976, for review). Our results, establishing a different cardenolide array in *A. syriaca* seeds collected in Connecticut than in seeds of *A. syriaca* collected in Quebec, Ontario, and Ohio, and the differences in the numbers of cardenolides detected in the seeds of the different populations of *A. syriaca* used in the studies mentioned above, indicate that geographic variation also occurs in the seeds of *A. syriaca*.

In vivo and in vitro evidence with two nonasclepiad cardenolides, ouabain and digitoxin, indicates that differential excretion and metabolism of cardenolides in *O. fasciatus*, as well as preferential uptake of individual cardenolides across the gut and into the dorsolateral space, are involved in the selective sequestration of cardenolides in *O. fasciatus* (Scudder and Meredith, 1982b; Duffey et al., 1978; Meredith et al., 1984). However, ouabain and digitoxin do not occur in the host plants of *O. fasciatus*. The present study provides evidence for differential excretion and metabolism in *O. fasciatus* of cardenolides present in its natural food plants. The presence of many cardenolides in *O. fasciatus* that were not detected in its food sources suggests metabolism of seed cardenolides or concentration in the insect of seed cardenolides present in such low concentrations they were not detected by TLC analysis. Metabolism of seed cardenolides may occur in the seed while it is being digested by the saliva of the insect, in the insect gut by bacteria or the gut milieu, and/or in other tissues within the insect. Two other insect species are known to metabolize cardenolides ingested with their food plant (Seiber et al., 1980; Brower et al., 1982; Levey, 1983): in one of these, the monarch, homogenates of both the gut and fat body metabolized the asclepiad cardenolide, uscharidin (Marty and Krieger, 1984).

Very polar and very nonpolar cardenolides were absent or in very low concentrations in *O. fasciatus* reared on *A. speciosa* or *A. syriaca*. The differential excretion of large amounts of intermediate and higher polarity cardenolides relative to cardenolides of lower polarity in the urine + feces may explain, in part, the low levels of polar cardenolides in *O. fasciatus* when feeding upon seeds of *A. speciosa*. Rapid metabolism of nonpolar cardenolides in the insect may also explain the absence of these cardenolides in *O. fasciatus*. Seiber et al. (1980) have shown in the monarch that rapid metabolism of several less polar cardenolides to more polar metabolites results in the absence of or very low concentrations of these cardenolides in the larval tissue.

The sequestration and concentration of cardenolides of a wide polarity range in *O. fasciatus* reared on *A. syriaca* or *A. speciosa* suggests that cardenolides are not sequestered in the insect simply on the basis of polarity. The importance of physical-chemical characteristics other than polarity in cardenolide sequestration in *O. fasciatus* is also indicated by the sequestration and concentration of intermediate and more polar cardenolides in the fat body of *O. fasciatus* reared on *A. speciosa*. This was unexpected since the fat body often accumulates nonpolar compounds and toxins owing to their lipophilic nature (Kilby, 1963), and earlier observations indicated preferential sequestration of the nonpolar cardenolide digitoxin in the fat body of adult *O. fasciatus* (Duffey et al., 1978). The importance of physical-chemical characteristics other than polarity in cardenolide sequestration has also been indicated in the monarch butterfly (Seiber et al., 1980; Brower et al., 1982), and Seiber et al., (1980) have suggested that physical-chemical characteristics that influence the chemical stability, solubility, and ease of transport of individual cardenolides and their binding affinity to blood proteins and sequestration systems in the insect are involved.

In summary, this study provides evidence that cardenolides are differentially distributed in *O. fasciatus* in terms of total quantity. In contrast, only minor differences were detected in the cardenolide array of adults and five tissues and secretions of *O. fasciatus*. This study also provides evidence that cardenolides of a wide polarity range can be sequestered in *O. fasciatus;* however, very nonpolar and very polar cardenolides are not sequestered or are present in extremely low concentrations. We have also shown that metabolism and differential excretion of cardenolides are part of the selective sequestration process of cardenolides in *O. fasciatus*. Finally, the constancy of the cardenolide profiles of *O. fasciatus* reared on seeds of two species of milkweed with very different cardenolide arrays, and between tissues of *O. fasciatus* reared on a single species of milkweed seeds, indicates that there is qualitative regulation of the cardenolide array in *O. fasciatus*, as in the monarch (Brower et al., 1982).

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# OVIPOSITION AND FEEDING PREFERENCES OF THE SOUTHERN PINE CONEWORM (LEPIDOPTERA: PYRALIDAE) FOR DIFFERENT HOST-PLANT MATERIALS AND OBSERVATIONS ON MONOTERPENES AS AN OVIPOSITION STIMULANT[1]

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**Abstract**—Bioassays were conducted with first-stage larvae and newly emerged females of the southern pine coneworm, *Dioryctria amatella* (Hulst), to detect feeding and ovipositional preferences for different host-plant materials collected between April and August. Correlations between measures of insect preferences and estimates of wood parameters indicated that larvae preferred to feed on host-plant materials with high moisture contents, low wood densities, and low monoterpene contents. Female moths, however, did not necessarily select those plant materials preferred by larvae for feeding, but tended to oviposit more frequently on materials with high monoterpene contents. A synthetic mixture of monoterpenes similar in composition to those present in fusiform rust galls of slash pine (*Pinus elliottii*) Englem. var. *elliottii*) elicited mating and oviposition behavior.

**Key Words**—Southern pine coneworm, *Dioryctria amatella* (Hulst), Lepidoptera, Pyralidae, oviposition and feeding preference, synthetic oviposition stimulant, slash pine, *Pinus elliottii* Engelm. var. *elliottii*, monoterpenes, fusiform rust, *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme.*

## INTRODUCTION

Feeding habitats of the southern pine coneworm, *Dioryctria amatella* (Hulst), include female and male strobili and shoots of southern pine, *Pinus* spp., galls caused by the fusiform rust fungus, *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme*, and strobili infected with the southern cone rust, *Cronar-*

[1] Mention of proprietary products does not necessarily imply endorsement by USDA.

*tium strobilinum* (Arth.) Hedgc. and Hahn, throughout the range of the insect (Ebel 1963, 1965; Neunzig et al., 1964; Coulson and Franklin, 1970; Ebel et al., 1975). The relative abundance of the southern pine coneworm (SPC) in these habitats, however, varies considerably throughout the year.

Neunzig et al. (1964) found infestations of the SPC to be common in rapidly elongating shoots of longleaf pine (*Pinus palustris* Mill.) in the spring in North Carolina, but by late June the infestations were most abundant in rapidly growing second-year strobili. Ebel (1965) also reported seasonable shifts in larval habitat of the SPC between different parts of slash pine (*P. elliottii* Englem. var. *elliottii*) and longleaf pine in north Florida. During January and February young larvae infested the rapidly expanding first-year strobili and then infested the expanding shoots in March and April. First-year strobili infected with the southern cone rust and healthy second-year strobili were commonly attacked from late spring to early summer. Larvae were present in fusiform rust galls throughout the year.

The SPC is multivoltine and in north Florida may have up to four generations per year (Ebel, 1965; Merkel and Fatzinger, 1971). The sites upon which moths deposit eggs in the field are largely unidentified, but Ebel (1965) observed single egg shells of the SPC on scale leaves of male strobilus buds. In laboratory cages, the females deposit eggs singly, and the fecundity of gravid females is highly variable (Ebel, 1965; Merkel and Fatzinger, 1966; Fatzinger, 1981). Some evidence indicates that adult coneworms are responsive to monoterpenes found in slash pines (Asher, 1970) and that monoterpenes may serve as oviposition stimulants for the SPC. Mating and oviposition by the SPC were dependent on the presence of host-plant material (a fusiform rust gall) in laboratory rearings, even when the moths were separated from the plant material by layers of cloth (Fatzinger, 1981). Under these conditions, volatile plant chemicals such as monoterpenes could be reponsible for eliciting oviposition responses by the caged moths. Infestations of the SPC in bark areas adjacent to naval stores wounds or near other bark wounds on slash and longleaf pines (Fatzinger and DeBarr 1969) may also be evidence for a preference of moths to deposit eggs near concentrated sources of monoterpenes. Variations in the concentrations of monoterpenes might accompany changes in the growth rates of plant materials and result in oviposition preferences for different materials throughout the year. Such differences could then account for seasonal shifts in feeding habitats of larvae.

Our objectives were to determine: (1) whether seasonal shifts in larval feeding habitats on slash pines could be explained by periodic changes in SPC oviposition preferences for the different plant materials, (2) the relationships of three physical properties (monoterpene contents, moisture contents, and wood densities) of plant materials to SPC feeding and oviposition preferences, and (3) the role of monoterpenes in the selection of plant materials by SPC for oviposition. We compared the feeding responses of newly hatched larvae with the oviposition responses of moths to four types of host-plant materials collected

three different times during a year. Estimates of the three physical properties of different plant materials were also compared with oviposition and feeding responses to evaluate their influences on SPC preferences. Subsequent bioassays were conducted to determine the effect of a synthetic mixture of monoterpenes on the oviposition behavior of the SPC.

## METHODS AND MATERIALS

Bioassays were conducted during late April, early June, and mid-August to detect seasonal differences in preferences of first-stage larvae and newly emerged female moths for four categories of host plant material: (1) rapidly growing materials in which larvae are periodically most abundant [shoots in late April or second-year female strobili (cones) collected in early June or mid August], (2) slowly growing first-year female strobili (conelets) in which larvae are more abundant earlier in the year, (3) healthy branches in which larvae are rarely found, and (4) fusiform rust galls which larvae infest throughout the year. Estimates of tissue edibility, succulence (wood density or moisture content), and concentrations of possible olfactory stimulants (extractive yields of monoterpenes) were also compared with overt SPC preferences for each category of material during late April and early June.

Samples of the four categories of plant materials were collected from slash pines in baker County, Florida, sealed in plastic bags, and held in a refrigerator (8 °C) for up to seven days until used in bioassays. Additional samples collected for chemical analyses were stored in a freezer (−5 °C) for up to 14 days. Female strobili were collected intact, but shoots and branches were cut to a length of ca. 10 cm. The cut ends of plant materials used for bioassays were sealed with melted paraffin wax within 5 min of collection. All samples in categories 1–3 were collected from four trees that had previous records of numerous attacks by SPC on female strobili. Fusiform rust galls (category 4) were collected at random from branches of young slash pines. Each gall was cut into four transverse sections of approximately equal length, and each section was assigned at random to a different test.

Wood densities, moisture contents, and extractive yields of monoterpenes were determined from 50-g samples of each category by methods similar to those of Drew et al. (1971), Anon. (1976, 1982), and Kossuth and Munson (1980). Average values of the wood parameters were determined on a unit volume basis (Franklin and Squillace, 1974) from three replicate samples per tree and one sample per gall. Comparisons between insect preferences and wood parameters were made for sample materials collected from the same trees or galls.

SPC were obtained from a laboratory colony reared on artificial diet (Fatzinger, 1981) to prevent possible preconditioning of preferences for host-plant

materials. Individually caged moths or larvae were presented with a pair of host-plant materials and the proportions of eggs deposited, or larvae feeding, on the different materials were used as criteria of preferences. SPC that died before feeding or ovipositing on the plant materials were excluded from the results because they did not provide data for estimating preferences for the paired plant materials. Moths that deposited fewer than 15 eggs were also excluded from the results because the oviposition rates were considered to be too low for comparisons of biological significance. The effects of interactions between moth preferences for chemical stimuli and preferences for surface textures (Callahan, 1957) were eliminated by covering all plant materials in the oviposition tests with two layers of cheesecloth to provide a uniform surface texture for egg deposition.

A bioassay consisted of eight replicate cages of moths and 40 replicate cages of larvae for each of the six combinations of paired plant materials. Tests of larval preferences were initiated by placing a larva and two test materials in each cage (1-qt-size glass jar fitted with a cloth lid) and were terminated after the larva began feeding continuously and burrowed into one of the plant materials. Ovipositional preferences were tested in wire screen cages (30 × 30 × 30 cm) containing one female plus two male moths, and a sponge saturated with a 5% solution of sugar water for moth feeding. Egg deposition was recorded and plant materials were replaced with fresh ones at ca. three-day intervals. These tests were continued until the female moths died, but the sex ratio within cages was maintained at ca. 0.5 by replacing dead male moths with newly emerged males. The 240 larvae, 48 female moths, and 96 male moths required for each bioassay were usually obtained over a period of several days. Sets of six cages of larvae or moths, each containing a different pair of host plant materials, were initiated daily until all replicates were obtained.

The percentage of monoterpene constituents and microliters of monoterpenes extracted per gram of green wood were also compared between pairs of healthy branches and fusiform rust galls collected from branches on 30 trees in February and 29 trees in June. A pair consisted of the entire galled area excised from one branch and a section of a healthy branch collected on the opposite side of the tree at the same height above ground. Prior to extraction, each material was ground to a coarse powder and 50 $\mu$l of paracymene were added per 50 g of material as an internal standard for subsequent use in gas–liquid chromatography (GLC). Monoterpenes were analyzed on a dual column Hewlett-Packard model 5840A gas chromatograph equipped with flame ionization detectors. The monoterpene extracts were diluted 1:2000 (v/v) with $n$-pentane and ca. 1 $\mu$l was injected onto 6-m long × 3.2-mm OD copper columns packed with 20% Carbowax 20 M on 80/100 Chrom W. Operating parameters for the analyses were: injector temperature 175°C, oven temperature 120°C, detector temperature 300°C, and carrier gas flow (helium) 25.0 ml/min.

A mixture of monoterpenes was prepared in approximately the same pro-

TABLE 1. MONOTERPENE CONSTITUENTS OF A SYNTHETIC OVIPOSITION STIMULANT FOR SOUTHERN PINE CONEWORM BASED ON AVERAGE YIELDS OF EXTRACTIVES FROM FUSIFORM RUST BRANCH GALLS OF SLASH PINE

| Monoterpene | Average % composition of galls | Synthetic mixture[a] Amount (ml) | Synthetic mixture[a] Composition (%) |
|---|---|---|---|
| α-Pinene | 46.6 | 95.0 | 47.5 |
| Camphene | 1.1 | 0 | 1.2 |
| β-Pinene | 41.3 | 84.0 | 41.6 |
| Myrcene | 1.6 | 1.0 | 1.8 |
| α-Phellandrene | 0.8 | 3.2 | 0.8 |
| Limonene | 2.1 | 0 | 4.9 |
| β-Phellandrene | 6.5 | 20.0 | 3.2 |

[a]The volumes of reagent monoterpenes mixed were adjusted to account for contaminant monoterpenes found present by GLC.

portions found in fusiform rust galls to determine whether monoterpenes alone could act as an oviposition stimulant for the SPC (Table 1). The mixture was diluted with paraffin oil to produce a final concentration equivalent to the average total microliters of monoterpenes extracted per gram of gall (green weight), which was equal to 0.14 g of the synthetic mixture per cubic centimeter of paraffin oil. The synthetic mixture was bioassayed by placing 20 ml in a stendor dish covered with two layers of cheesecloth on which moths could deposit eggs. The synthetic mixture was evaluated as an oviposition stimulant by comparing the numbers of eggs deposited per female in 96 cages containing galls and 27 cages containing the synthetic mixture, and the proportions of eggs deposited in 33 cages each containing one gall and one container of the synthetic mixture.

Results of biossays with paired materials were analyzed with one-sample $t$ tests for the null hypothesis $H_0$: $\mu_1 = 0.5$, where $\mu_1$ was the proportional response of SPC for one of the paired materials. Differences among other treatment means were tested for statistical significance by analysis of variance and Duncan's multiple-range test. Prior to analyses, data recorded as proportions or percentages were transformed to arcsin $\sqrt{\text{proportion}}$, but only untransformed proportions and percentages are listed in the tables and results.

## RESULTS AND DISCUSSION

*Preferences for Plant Material.* First-year conelets were generally the least preferred substrate tested for feeding or oviposition by the SPC under the conditions imposed during bioassays (Tables 2 and 3). This response to conelets

TABLE 2. RESPONSES OF FEMALE MOTHS OF SOUTHERN PINE CONEWORM TO DIFFERENT PAIRS OF HOST-PLANT MATERIALS COLLECTED FROM SLASH PINES AND TESTED AS SUBSTRATES FOR OVIPOSITION

| Time of substrate collection | No. of cages with eggs | No. eggs deposited | | Average proportions of eggs per substrate pair[a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | On the substrates | At random | Gall | Branch | Conelet | Shoot | Cone |
| Late April | 8 | 1210 | 38 | 0.72 | 0.28 | | | |
| | 7 | 1155 | 30 | 0.87 | | 0.13* | | |
| | 7 | 1339 | 93 | 0.77 | | | 0.23 | |
| | 6 | 483 | 18 | | 0.68 | 0.32* | | |
| | 7 | 702 | 143 | | 0.32 | | 0.68 | |
| | 6 | 330 | 19 | | | 0.41 | 0.59 | |
| Early June | 8 | 848 | 48 | 0.41 | 0.59 | | | |
| | 7 | 398 | 25 | 0.82 | | 0.18 | | |
| | 5 | 408 | 46 | 0.49 | | | | 0.51 |
| | 6 | 367 | 32 | | 0.82 | 0.18* | | |
| | 7 | 891 | 20 | | 0.62 | | | 0.38 |
| | 7 | 564 | 94 | | | 0.12 | | 0.88* |
| Mid-August | 8 | 1041 | 100 | 0.52 | 0.48 | | | |
| | 5 | 668 | 10 | 0.97 | | 0.03** | | |
| | 8 | 827 | 20 | 0.72 | | | | 0.28 |
| | 7 | 609 | 61 | | 0.87 | 0.13* | | |
| | 6 | 309 | 37 | | 0.45 | | | 0.55 |
| | 8 | 523 | 28 | | | 0.15 | | 0.85* |

[a]Significance of differences between treatment means is indicated by asterisks (* = $P < 0.05$, ** = $P < 0.01$).

collected during or after late April is consistent with Ebel's (1965) observation that first-year strobili are frequently inhabited by larvae only during January and February in north Florida. After this initial period of rapid strobilus growth, larvae apparently migrate to other rapidly growing host materials such as shoots or first-year strobili infected with the southern cone rust.

Significant differences were not found between SPC preferences for healthy and gall-infected branches with the exception that larvae showed a preference for feeding on healthy branches collected in early June (Table 3). There appeared to be an interaction, however, between months and preferences for galls and branches, with moths and larvae tending to prefer galls over branches in late April and branches over galls in early June. The proportions of eggs deposited on branches or shoots collected in late April were not significantly different (Table 2), but larvae fed more frequently on the shoots than on branches (Table 3). Thus, shoot infestations during the spring may be a result of the feeding preferences of migrating larvae rather than moth ovipositional preferences.

TABLE 3. RESPONSES OF FIRST-STAGE LARVAE OF SOUTHERN PINE CONEWORM TO DIFFERENT PAIRS OF HOST-PLANT MATERIALS COLLECTED FROM SLASH PINES AND TESTED AS SUBSTRATES FOR FEEDING

| Time of substrate collection | No. of cages in test | No. of larvae that fed on substrates | Average proportions of larvae that fed per substrate pair[a] | | | | |
|---|---|---|---|---|---|---|---|
| | | | Gall | Branch | Conelet | Shoot | Cone |
| Late April | 38 | 23 | 0.68 | 0.32 | | | |
| | 40 | 34 | 0.75 | | 0.25 | | |
| | 40 | 32 | 0.30 | | | 0.70 | |
| | 38 | 25 | | 0.79 | 0.21 | | |
| | 40 | 37 | | 0.11 | | 0.89* | |
| | 40 | 32 | | | 0.07 | 0.93* | |
| Early June | 40 | 25 | 0.32 | 0.68* | | | |
| | 40 | 17 | 0.44 | | 0.56 | | |
| | 40 | 20 | 0.21 | | | | 0.79 |
| | 40 | 21 | | 0.68 | 0.32 | | |
| | 40 | 19 | | 0.30 | | | 0.70 |
| | 40 | 18 | | | 0.18 | | 0.82 |
| Mid-August | 40 | 28 | 0.57 | 0.43 | | | |
| | 40 | 33 | 0.94 | | 0.06* | | |
| | 40 | 32 | 0.16 | | | | 0.84* |
| | 40 | 31 | | 0.92 | 0.08* | | |
| | 40 | 36 | | 0.36 | | | 0.67 |
| | 40 | 35 | | | 0.11 | | 0.89* |

[a]Significance of differences between treatment means is indicated by asterisks (* = $P < 0.05$).

Moths oviposited about equal numbers of eggs on second-year cones, galls, and branches collected in early June and mid-August (Table 2), but larvae fed more frequently on second-year cones than on galls collected in mid-August (Table 3).

*Preferences for Physical Properties of Plant Materials.* The degree of correlation between moth and larval preferences for given host-plant materials was weak ($r = 0.32$).[2] Larvae tended to show feeding preferences for the more succulent plant materials, i.e., feeding was positively correlated with moisture content ($r = 0.10$) and negatively correlated with wood density ($r = -0.23$*) or monoterpene content ($r = -0.30$*). Materials with high moisture contents, low monoterpene contents, and low wood densities were generally the most

[2]Significance of correlations is indicated by asterisks (*$P < 0.05$, ** = $P < 0.01$). The probabilities of correlations not followed by an asterisk were $P > 0.05$.

TABLE 4. COMPARISONS OF AVERAGE MONOTERPENE CONTENTS, MOISTURE CONTENTS, AND WOOD DENSITIES OF SLASH PINE MATERIALS USED DURING BIOASSAYS OF FEEDING AND OVIPOSITION PREFERENCES FOR SOUTHERN PINE CONEWORM

| Time of substrate collection | Plant material[a] | Monoterpene content[b] (g/cc) | Moisture content[b] (g/cc) | Wood density[b] (g/cc) |
|---|---|---|---|---|
| Late April | Gall | 0.14a | 0.31a | 0.39a |
| | Conelet | 0.08b | 0.68b | 0.27b |
| | Branch | 0.05c | 0.54c | 0.38a |
| | Shoot | 0.05c | 0.65b | 0.23c |
| Early June | Gall | 0.12a | 0.35a | 0.42a |
| | Conelet | 0.08b | 0.68b | 0.29b |
| | Branch | 0.05bc | 0.50c | 0.41a |
| | Cone | 0.04c | 0.73d | 0.22c |

[a] Gall = fusiform rust branch gall, branch = healthy branch, conelet = first-year female strobilus, cone = second-year female strobilus, shoot = rapidly elongating shoot.
[b] Any two means followed by the same letter are not significantly different at the 5% probability level.

rapidly growing plant materials at the time of collection, e.g., shoots collected in late April or cones collected in early June (Table 4). Moths, however, did not show particular preferences for ovipositing on the more rapidly growing plant materials collected during any of the bioassay periods. Oviposition was positively correlated with monoterpene content ($r$ = 0.14) and wood density ($r$ = 0.24*), but negatively correlated with moisture content ($r$ = −0.36**). Thus, moths did not necessarily select those plant materials for oviposition that were preferred by larvae for feeding. Larval preferences for moist, succulent plant materials were also noted by Ebel (1965) who observed larval migrations from dead, dry host plant materials to fresh, green materials both in the field and in laboratory colonies. The relationship between oviposition preferences and higher concentrations of monoterpenes might explain, in part, the insect's selection of fusiform rust galls for oviposition throughout the year (Tables 2 and 4).

*Monoterpenes Extracted from Galls.* Significant differences in extractive yields of individual and total monoterpenes were found between paired samples of healthy and galled branch sections (Table 5). The total volumes of monoterpenes did not differ significantly between the February and June collections of galls or branches. Extracts from galls tended to have higher percentages of $\alpha$-pinene, camphene, and $\alpha$-phellandrene, and lower percentages of $\beta$-pinene, myrcene, and $\beta$-phellandrene than did extracts from healthy branches (Table 5). Average yields of monoterpenes extracted during February and June were about five times greater from galls (10.9 $\mu$l/g) than from healthy branches (2.1 $\mu$l/g).

TABLE 5. YIELDS OF MONOTERPENES EXTRACTED FROM FUSIFORM RUST GALLS AND HEALTHY BRANCHES COLLECTED IN PAIRS FROM INDIVIDUAL SLASH PINE TREES IN BAKER COUNTY, FLORIDA

| | Average μl/g of green wood[a] | | | | Composition (%)[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | February | | June | | February | | June | |
| Monoterpene | Gall | Branch | Gall | Branch | Gall | Branch | Gall | Branch |
| α-Pinene | 4.48 | 0.88 | 6.42 | 0.81 | 45.0 | 43.3 | 48.2 | 40.2** |
| Camphene | 0.11 | 0.01 | 0.15 | 0.01 | 1.1 | 0.9** | 1.2 | 0.9* |
| β-Pinene | 3.76 | 0.91 | 4.77 | 0.92 | 41.6 | 42.7 | 41.0 | 44.8** |
| Myrcene | 0.16 | 0.05 | 0.17 | 0.04 | 1.8 | 2.4 | 1.4 | 2.5** |
| α-Phellandrene | 0.08 | <0.01 | 0.05 | <0.01 | 1.0 | 0.3* | 0.4 | 0.3** |
| Limonene | 0.22 | 0.03 | 0.23 | 0.05 | 2.4 | 1.5** | 1.8 | 2.6 |
| β-Phellandrene | 0.51 | 0.21 | 0.68 | 0.15 | 7.1 | 9.8* | 5.9 | 8.7* |
| Total | 9.36 | 2.12 | 12.49 | 2.02 | | | | |

[a] All means are significantly different ($P \leqslant 0.01$) between galls and branches.
[b] Significance of differences between treatment means is indicated by asterisks (* = $P < 0.05$, ** = $P < 0.01$).

*Synthetic Oviposition Stimulant.* Mating and oviposition occurred in cages containing only the synthetic mixture of monoterpenes (Table 1) diluted with paraffin oil to a concentration equivalent to that present in fusiform rust galls (Table 6). Although female moths deposited viable eggs on cheesecloth covers surrounding the synthetic mixture, they consistently deposited more eggs when galls were the only substrates. The average numbers of eggs deposited per female when both substrates were present in cages were significantly different ($P < 0.01$) between galls and the synthetic mixture.

TABLE 6. RESPONSES OF FEMALE MOTHS OF SOUTHERN PINE CONEWORM TO FUSIFORM RUST GALLS AND SYNTHETIC STIMULANT TESTED AS OVIPOSITION SUBSTRATES

| Test no. | No. females tested | Avg. no. eggs per substrate[a] | |
|---|---|---|---|
| | | Gall | Synthetic stimulant |
| 1 | 96 | 119.3 ± 20.6 | – |
| 2 | 27 | – | 66.1 ± 17.8 |
| 3 | 33 | 53.3 ± 9.9 | 35.5 ± 6.5 |

[a] Means ± standard errors ($P = 0.05$).

## CONCLUSIONS

A mixture of seven monoterpenes identified from fusiform rust galls of slash pine was found to serve as a synthetic oviposition stimulant for the SPC. Differences in rates of oviposition on galls and the synthetic mixture indicated that other volatile chemicals were present in galls, but absent from the synthetic mixture, that served as additional stimulants for oviposition. When given a choice, female moths tended to oviposit on host-plant materials with the highest concentrations of monoterpenes, and those materials were not necessarily the same ones preferred by first-stage larvae for feeding. Thus, the volatile monoterpenes may be used by females of the SPC primarily for locating suitable host trees for oviposition. The relatively high concentrations of monoterpenes near tree wounds or in rust galls, however, could result in increased rates of oviposition and account for the abundance of larvae at these sites throughout the year. In the absence of galls or wounds, however, random oviposition on host trees could explain why SPC eggs are rarely found on other plant materials that are frequently attacked by larvae. Variations in the susceptibilities of trees to attack by SPC (Merkel et al., 1965) could also result from differences in the concentrations of constituents or monoterpenes in individual trees. First-stage larvae generally selected plant materials with high moisture contents, low wood densities, and low monoterpene contents during bioassays of feeding preferences. The shifts in larval habitats may therefore be the result of larvae moving from oviposition sites, previously utilized habitats, or overwintering sites to the more succulent, rapidly growing plant tissues present on the host tree at different times of the year.

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# INSECTICIDAL CHROMENES FROM THE VOLATILE OIL OF *Hemizonia fitchii*[1,2]

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**Abstract**—Based on field observations of the effects of the resinous tarweed *Hemizonia fitchii* A. Gray (Asteraceae) on mosquito populations in California, the volatile oil of this plant was investigated for insecticidal activity. Analysis of the oil by TLC and capillary GC-MS showed the presence of five major constituents which were identified as the monoterpenoid 1,8-cineole, and the chromenes encecalin, eupatoriochromene (desmethylencecalin), 6-vinyl-7-methoxy-2,2-dimethylchromene, and desmethoxyencecalin. Trace amounts of several volatile fatty acids, alkanes, *p*-coumarate derivatives, additional chromene derivatives, and numerous mono- and sesquiterpenoids were also detected and identified by GC-MS. Fractionation of the oil by preparative TLC and column chromatography afforded the major chromenes, the identities of which were confirmed by NMR and IR spectral data. The chromenes exhibited weak to moderate toxicity against *Culex pipiens* (house mosquito) larvae and *Oncopeltus fasciatus* (large milkweed bug) nymphs. However, no anti-juvenile hormone activity was observed for any of the compounds tested against these insect species.

**Key Words**—*Hemizonia fitchii,* insecticidal volatile oil, chromenes, 1,8-cineole, *Culex pipiens, Oncopeltus fasciatus,* mosquito, milkweed bug.

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## INTRODUCTION

In a systematic search for naturally occurring compounds which exhibit effects on the growth, development, and behavior of insect pests, we are currently screening extracts from over 800 species of higher plants found in the western United States. The basis for the present report was a field observation by one of us (E.K.) that resinous *Hemizonia fitchii* A. Gray (Asteraceae) (Fitch's spikeweed, tarweed) growing at the edges of ponds in California had a definite suppressant effect on local mosquito populations. Pond water in contact with *H. fitchii* was observed to be totally devoid of any stage of mosquito. However, nearby ponds, which appeared to be identical chemically, physically, and biologically, with the exception that there was no contact with *H. fitchii,* supported large numbers of all stages of the mosquito *Aedes melanimon.* In addition, the aboveground parts of *H. fitchii* possess a strong, pungent, lachrymatory aroma that was observed in the field to be repellent to insects and spiders, but highly attractive to domestic cats.

Previous phytochemical studies on members of the genus *Hemizonia* have resulted in the identification of an acetylenic thiophene (Bohlmann et al., 1973), sesquiterpenes, diterpenes, benzofurans, benzopyrans (chromenes), *p*-coumarate derivatives, squalene, an alkanol ester, several geraniol esters (Bohlmann et al., 1981), and a number of flavonoids (Proksch et al., 1984). However, no biological studies appear to have been conducted previously with any species of *Hemizonia*.

## METHODS AND MATERIALS

*Extraction.* Aerial parts of *H. fitchii* were collected in June in Oroville, California, at Afterbay of the Feather River Power Water Project. Collected plants were sealed in plastic bags, frozen (−20°C), and airmailed to Salt Lake City, Utah. Upon arrival, steam distillates (2.50 g) were obtained from the frozen whole plant material (160 g, fresh weight) using a modified Clevenger distillation apparatus (24 hr). The plant material was subsequently dried, finely ground with a Wiley mill, and sequentially extracted with hexane, methylene chloride, and methanol. Since bioassay-guided fractionation using larvae of *Culex pipiens* (see below for a description of the bioassay) confirmed that the vast majority of the biological activity resided in the volatile oil fraction, further chromatographic work was confined to the steam distillate fraction.

*Gas-Liquid Chromatography (GC) of Volatile Oil.* GC analyses were performed with a Varian 1800 gas chromatograph equipped with a flame ionization detector (350°C) using a J & W DB-1 fused silica capillary column (30 m × 0.32 mm ID; 0.25 μm film thickness) with nitrogen as the carrier gas (18 cm/sec). All GC analyses were performed in the split mode (1:25 split ratio) with

the injector temperature at 275°C. The oven temperature was programed from 60° to 230°C at 4°C/min, and peak areas were calculated using a Columbia Scientific Industries Supergrator-2 electronic digital integrator.

*Thin-Layer (TLC) and Open-Column Chromatographies.* Analytical and preparative TLC were performed on 20 × 20-cm prescored silica gel GHLF plates (Analtech, Inc.; 0.25 mm) using hexane–diethyl ether–acetic acid (80 : 20 : 1, system A; multiple development) and hexane–diethyl ether [3 : 1 (B) and 4 : 1 (C)] as solvent systems. Visualization for analytical TLC was accomplished under long- and shortwave ultraviolet (UV) light, followed by spraying with a vanillin–sulfuric acid–ethanol (3 g : 1.5 ml : 100 ml) spray reagent and heating. For preparative TLC, visualization under UV light revealed the major bands which were subsequently cut from the plates and eluted with acetone.

Additional steam distillates from *H. fitchii* were subjected to column chromatography on silica gel 60 (30–70 mesh ASTM) in a gradient of ether in hexane. Separations were monitored by subjecting eluted fractions to analytical TLC with visualization under UV light, followed by spraying with the vanillin–sulfuric acid spray reagent and heating.

*Gas Chromatography–Mass Spectrometry (GC-MS) of Volatile Oil.* GC-MS analyses were performed with a Hewlett-Packard model HP-5985 quadrupole gas chromatograph–mass spectrometer, taking mass spectral scans from mass 33 to mass 633 at 800 amu/sec. Chromatographic separations were achieved using a J & W DB-1 fused silica capillary column (26 m × 0.32 mm ID; 1 $\mu$m film thickness). All GC-MS analyses were made using a 1 : 35 split ratio with helium as carrier gas (2 cc/min). The GC column was temperature programed from 70° to 300° at 5°C/min. Compounds were identified by EI (electron impact, 70 eV) mass spectrometry and by their order of elution and relative GC retention times. The identification of 1,8-cineole (I) and several of the trace constituents was aided by the compilations of Jennings and Shibamoto (1980) and Swigar and Silverstein (1981), as well as the EPA/NIH mass spectral data base (Heller and Milne, 1978, 1980).

*Proton Nuclear Magnetic Resonance ([$^1$H]NMR) Spectroscopy and Infrared (IR) Spectrophotometry of the Major Chromenes.* The identities of the major chromenes were confirmed by 60-MHz [$^1$H]NMR spectroscopy (Varian EM-360) using $CDCl_3$ or benzene-$D_6$ as solvent and tetramethylsilane (TMS) as internal standard. IR spectrophotometric data (Perkin-Elmer 710B) were consistent with the assigned structures in all cases.

Portions of the volatile oil were acetylated by using acetic anhydride with pyridine as catalyst. Acetylated compounds were separated on silica gel GHLF plates (Analtech, Inc.; 0.25 mm) using hexane–diethyl ether–acetic acid (80 : 20 : 1) as the solvent system. Identification of the acetates was accomplished with IR spectral data.

*Culex Larvicidal Assay.* A susceptible strain of *C. pipiens* originally obtained from the California State Department of Health was used for the larvi-

cidal assay. Animals (all stages of either first or third instar) were counted into test containers (1-oz plastic cups) and treated with a graduated concentration series of 0.1% acetone-diluted test compounds in 10 ml distilled water. First instar larvae were transferred (10 larvae/cup) with a fine-mesh silk cloth. Third instar larvae were transferred (5 larvae/cup) with a 1 × 1-in. circle of ordinary window screen. Care was taken to remove excess water before entering the larvae into the test solutions. Following 48 hr of exposure to the treated water at 28°C, 80% relative humidity, and 18 hr daily illumination, $LC_{50}$ values, the lethal concentrations for 50% mortality, were estimated using log probit paper. The assay was repeated three times with four treatments using 5–10 larvae/treatment. Survivors were allowed to complete development in order to observe any developmental effects of sublethal concentrations.

*Oncopeltus Topical Assay.* Nymphs of the large milkweed bug, *O. fasciatus,* were taken from a laboratory culture maintained on sunflower seeds and water. Animals (all stages of either second or third instar) were temporarily anesthetized with $CO_2$ and topically treated on the dorsum of the abdomen with 1 $\mu$l of an acetone solution of the test compound. The treated insects were transferred to rearing jars with sunflower seeds and water at 28°C, 80% relative humidity, and 18 hr daily illumination for the 8–10 days' duration of the assay period (sufficient time for control insects to undergo two molts). Appropriate controls were kept for each of the treated groups. $LD_{50}$ values, the lethal doses for 50% mortality, were estimated using log probit paper. The assay was repeated three times with four treatments using 5–10 nymphs/treatment. Survivors were allowed to complete development so that any developmental effects of sublethal doses could be observed.

## RESULTS

Because of the highly resinous and aromatic nature of the plant material, aerial parts of *H. fitchii* were subjected to steam distillation (24 hr), which afforded a viscous yellow volatile oil. The plant material was then dried, finely ground, and sequentially extracted with hexane, methylene chloride, and methanol. Bioassay of the various extracts with larvae of *C. pipiens* revealed that the vast majority of the biological activity resided in the volatile oil fraction.

Analysis of the volatile oil by TLC and fused silica capillary GC revealed the presence of only five major components. These major constituents were identified by fused silica capillary GC-MS as the monoterpenoid 1,8-cineole (I) (which comprised approximately 25% of the oil), and the chromenes encecalin (II) (the major constituent of the oil at 30%), eupatoriochromene (desmethylencecalin) (III) (17% of the oil), 6-vinyl-7-methoxy-2,2-dimethylchromene (IV) (7% of the oil), and desmethoxyencecalin (V) (6% of the oil) (Figure 1). These five major constituents together accounted for approximately 86% of the oil.

FIG. 1. Structures of the major constituents of the volatile oil of *Hemizonia fitchii* (I–V), and the internally hydrogen-bonded form of III (VI).

The remainder of the oil consisted of a large number of constituents which were present in concentrations of approximately 1% or less. These trace constituents were also detected and identified by GC-MS and included several volatile fatty acids, alkanes, *p*-coumarate derivatives, additional chromene derivatives, and numerous mono- and sesquiterpenoids (Table 1).

Fractionation of the oil by preparative TLC and column chromatography afforded 1,8-cineole (I) and the major chromenes, the identities of which were established by [$^{1}$H]NMR and IR spectroscopies, as well as by direct probe MS. The spectral data and physical properties of the compounds isolated agreed with literature values in all cases (Bjeldanes and Geissman, 1969; Anthonsen, 1969; Bohlmann and Grenz, 1970, 1977; Steelink and Marshall, 1979; Swigar and Silverstein, 1981; Proksch and Rodriguez, 1982).

Only chromene III yielded a monoacetate derivative, confirming it to be the only compound bearing a hydroxyl group. However, this hydroxyl group was barely observed in the IR spectrum of eupatoriochromene (III). In addition, the hydroxyl proton was at 12.87 ppm in the [$^{1}$H]NMR spectrum of III, and it took

TABLE 1. ANALYSIS OF VOLATILE OIL OF *HEMIZONIA FITCHII* BY GC-MS[a]

| Compound | $M^{+}$ (*m/z*) | $R_t$ (min) | Peak area (% of total) |
|---|---|---|---|
| Volatile fatty acids | | | |
| Isobutyric acid | 88 | 2.75 | 0.26 |
| Isovaleric acid | 102[b] | 4.22 | 0.11 |
| 2-Methylbutyric acid | 102[b] | 4.63 | 0.19 |
| Monoterpenes | | | |
| Sabinene | 136 | 7.62 | 0.20 |
| Unidentified | 120[c] | 8.00 | 0.21 |
| *p*-Cymene | 134 | 8.87 | 0.14 |
| 1,8-Cineole (I) | 154 | 9.18 | 25.07 |
| Linalool | 154[b] (136[c]) | 10.88 | 0.65 |
| α-Terpineol isomer | 154[b] (136[c]) | 12.77 | 0.25 |
| Unidentified | 168[c] | 12.90 | 0.62 |
| *p*-Cymen-9-ol | 150 | 13.12 | 0.35 |
| Naphthalene[d] | 128 | 13.22[e] | 0.39[e] |
| 4-Terpineol | 154 | | |
| Unidentified | 136[c] | 14.13 | 0.20 |
| Unidentified | 142[c] | 14.38 | 0.40 |
| Unidentified | 198[c] | 15.75 | 0.27 |
| *p*-Cymen-7-ol (cuminyl alcohol) | 150 | 16.07 | 0.29 |
| Sesquiterpenes | | | |
| β-Caryophyllene[f] | 204[b] (189[c]) | 20.45 | 0.31 |
| Farnesol or isomer[f] ($C_{15}H_{26}O$) | 222 | 23.08 | 0.53 |
| Farnesol or isomer[f] ($C_{15}H_{26}O$) | 222 | 23.20 | 0.30 |
| Nerolidol or isomer[f] ($C_{15}H_{26}O$) | 222[b] (204[c]) | 23.58 | 1.08 |
| Unidentified | 150[c] | 24.28 | 0.28 |
| Chromenes | | | |
| Desmethoxyencecalin (V) | 202 | 25.37 | 6.47 |
| 6-Vinyl-7-methoxy-2,2-dimethylchromene (IV) | 216 | 26.00 | 7.42 |
| Encecalin isomer[f] | 232 | 26.80 | 0.27 |
| Evodionol isomer[f] | 248 | 26.90 | 0.45 |
| Eupatoriochromene (Desmethylencecalin) (III) | 218 | 28.14 | 17.34 |
| Encecalol isomer[f] | 234 | 28.78 | 1.12 |
| Encecalin (II) | 232 | 30.27 | 29.63 |
| Miscellaneous constituents | | | |
| *trans*-Methyl coumarate-*p*-dimethyl allyl ether | 246 | 32.10 | 0.15 |
| Palmitic acid | 256 | 32.32 | 1.30 |
| *cis*-Methyl coumarate-*p*-dimethyl allyl ether | 246 | 34.05 | 0.64 |
| Unidentified | 168[b,c] | 34.77 | 1.01 |
| Alkanes | | | |
| Pentacosane ($n$-$C_{25}H_{52}$) | 352 | 42.25 | 0.77 |
| Heptacosane ($n$-$C_{27}H_{56}$) | 380 | 45.30 | 0.74 |
| Nonacosane ($n$-$C_{29}H_{60}$) | 408[b] | 48.18 | 0.19 |

[a] See text for GC-MS conditions.
[b] Molecular ion not observed by EI-MS.
[c] Highest mass ion observed.
[d] Unlikely as a natural product; probably an isolation artifact or impurity.
[e] Unresolved mixture.
[f] Tentative compound identification based on MS and relative $R_t$ data (authentic standards not available).

18 min of vigorous shaking with deuterium oxide to completely exchange this proton. Unexpectedly, the acetate ester derivative of III appeared to be more polar than its parent compound, as shown by its lower $R_f$ value (0.0) in the TLC systems used. This finding, together with the IR and [$^1$H]NMR spectral evidence, confirmed that the hydroxyl group in III is strongly internally hydrogen-bonded. This gives the compound the character of an ether-like "third ring," making it less polar than encecalin (II) and desmethoxyencecalin (V) (see Figure 1, VI). Similar chromatographic behavior has been noted with the strongly internally hydrogen-bonded proton of plumbagin (Kubo et al., 1983).

*1,8-Cineole (I).* $R_f$ (system C) 0.60 (no color observed under longwave UV light; light blue color after spraying with vanillin–sulfuric acid reagent and heating); MS, *m/z* 154 ($M^{\ddagger}$, $C_{10}H_{18}O$, 67%), 139 ($M-CH_3$, 51), 136 ($M-H_2O$, 10), 125 (15), 121 (11), 111 (78), 108 (98), 96 (40), 93 (68), 84 (70), 81 (100), 71 (66), 69 (49), 55 (34), 43 (79).

*Encecalin (II).* $R_f$ (C) 0.23 (bright blue under longwave UV light; bright red after spraying with vanillin–sulfuric acid reagent and heating); IR, $\nu_{max}$ (neat, NaCl plates) 3050, 2975, 1660, 1605, 1285 $cm^{-1}$; MS, *m/z* 232 ($M^{\ddagger}$, $C_{14}H_{16}O_3$, 17%), 217 ($M-CH_3$, 100), 187 ($217-OCH_2$, 10), 185 ($217-CH_3OH$, 7), 174 ($217-CH_3CO$, 8), 145 (4), 144(4), 115(6), 101 (9); [$^1$H]NMR (60 MHz, $CDCl_3$)$\delta$ 1.43 (6H, s, $2-CH_3$), 2.56 (3H, s, $-COCH_3$), 3.88 (3H, s, $-OCH_3$), 5.59 (1H, d, $J_{3,4}$ = 10, H$-$3), 6.36 (1H, d, $J_{4,3}$ = 10, H$-$4), 6.46 (1H, s, H$-$8), 7.62 (1H, s, H$-$5), (benzene-$d_6$)$\delta$ 1.32 (6H, s, $2-CH_3$), 2.53 (3H, s, $-COCH_3$), 3.37 (3H, s, $-OCH_3$), 5.32 (1H, d, $J_{3,4}$ = 10, H$-$3), 6.21 (1H, d, $J_{4,3}$ = 10, H$-$4), 6.36 (1H, br s, H$-$8), 7.87 (1H, s, H$-$5).

*Eupatoriochromene (desmethylencecalin) (III).* $R_f$ (C) 0.38 (yellow-green under longwave UV light; light blue after spraying with vanillin–sulfuric acid reagent and heating); IR, $\nu_{max}$ (KBr) 3050, 2920, 1630, 1370 $cm^{-1}$; MS, *m/z* 218 ($M^{\ddagger}$, $C_{13}H_{14}O_3$, 26%), 203 ($M-CH_3$, 100), 185 ($203-H_2O$, 20), 160 ($203-CH_3CO$, 4); [$^1$H]NMR (60 MHz, $CDCl_3$)$\delta$ 1.43 (6H, s, $2-CH_3$), 2.53 (3H, s, $-COCH_3$), 5.62 (1H, d, $J_{3,4}$ = 10, H$-$3), 6.32 (1H, d, $J_{4,3}$ = 10, H$-$4), 6.37 (1H, br s, H$-$8), 7.37 (1H, s, H$-$5), 12.87 (1H, $D_2O$-exchangeable, s, $-$OH, intramolecularly H-bonded).

*6-Vinyl-7-methoxy-2,2-dimethylchromene (IV).* $R_f$ (C) 0.73 (no color observed under longwave UV light; purple color after spraying with vanillin–sulfuric acid reagent and heating); IR, $\nu_{max}$ (neat, NaCl plates) 3060, 2975, 1735, 1630, 1620 (C=C), 1500, 1295, 1130 $cm^{-1}$; MS, *m/z* 216 ($M^{\ddagger}$, $C_{14}H_{16}O_2$, 23%), 201 ($M-CH_3$, 100), 185 ($M-OCH_3$, 20), 158 ($185-CH_2{=}CH$, 5); [$^1$H]NMR (60 MHz, $CDCl_3$)$\delta$ 1.45 (6H, s, $2-CH_3$), 3.87 (3H, s, $-OCH_3$), 5.19 (1H, dd, $J_{cis}$ = 11, $J_{gem}$ = 2, H$-$12), 5.58 (1H, d, $J_{3,4}$ = 10, H$-$3), 5.65 (1H, dd, $J_{trans}$ = 18, $J_{gem}$ = 2, H$-$12), 6.37 (1H, d, $J_{4,3}$ = 10, H$-$4), 6.47 (1H, s, H$-$8), 7.05 (1H, dd, $J_{trans}$ = 18, $J_{cis}$ = 11, H$-$11), 7.20 (1H, s, H$-$5).

*Desmethoxyencecalin (V).* $R_f$ (C) 0.28 (no color observed under longwave

TABLE 2. 48-HOUR *CULEX PIPIENS* LARVICIDAL BIOASSAY

| Compound tested | Instar tested | $LC_{50}$ (ppm) |
|---|---|---|
| 6-Vinyl-7-methoxy- | 1st | 1.8 |
| 2,2-dimethylchromene (IV) | 3rd | 3.8 |
| Encecalin (II) | 1st | 3.0 |
| | 3rd | 6.6 |
| Eupatoriochromene (III) | 1st | 6.4 |
| | 3rd | 13.0 |
| 1,8-Cineole (I) | 3rd | [a] |

[a] No effect observed to 20 ppm.

UV light); MS, *m/z* 202 ($M^{+}$, $C_{13}H_{14}O_2$, 10%), 187 ($M-CH_3$, 100), 171 ($M-OCH_3$, 1), 158 (187-29, 1), 144 ($187-CH_3CO$, 17), 128 (2), 115 (9).

The toxicities of the major components isolated from the volatile oil to *C. pipiens* (house mosquito) larvae are shown in Table 2. The activity of each of the components was about twofold more against first instar than against third instar larvae. Thus, the most active of the compounds tested, 6-vinyl-7-methoxy-2,2-dimethylchromene (IV), had an $LC_{50}$ value of 1.8 ppm against first instar and 3.8 ppm against third instar larvae. The activity against first and third instar larvae of encecalin (II) was $LC_{50}$ = 3.0 ppm and 6.6 ppm, respectively, and that of eupatoriochromene (III) was $LC_{50}$ = 6.4 ppm and 13.0 ppm, respectively. Survivors were allowed to continue development to the adult stage. Although more deaths occurred with time, no effects were observed on development to subsequent larval stadia, pupal formation, or adult emergence.

Three commercially available volatile organic acids which we identified in the volatile oil, namely isovaleric, isobutyric, and 2-methylbutyric acids, were also assayed against third instar *C. pipiens* larvae. We found no activity of these acids to concentrations as high as 250 ppm.

The results of an additional assay of the *Hemizonia* chromenes with *O. fasciatus* (large milkweed bug) nymphs are shown in Table 3. In this assay, encecalin (II) was found to be the most active compound. Topical applications of encecalin (II) caused 50% mortality to second and third instar nymphs at 10 μg and 11 μg, respectively. 6-Vinyl-7-methoxy-2,2-dimethylchromene (IV) had $LD_{50}$ values against second and third instar nymphs of 23 μg and 35 μg, respectively. Eupatoriochromene (III) had no effect on second and third instar nymphs at concentrations up to 100 μg and 200 μg, respectively. Desmethoxyencecalin (V) was not tested due to insufficient quantities available. Survivors were allowed to continue development to the adult stage. Although more deaths occurred with time, no effects were observed on development to subsequent stadia, number of stadia, or adult emergence.

TABLE 3. *ONCOPELTUS FASCIATUS* TOPICAL ASSAY[a]

| Compound tested | Instar tested | $LD_{50}$ (μg) |
|---|---|---|
| Encecalin (II) | 2nd | 10 μg |
| | 3rd | 11 μg |
| 6-Vinyl-7-methoxy- | 2nd | 23 μg |
| 2,2-dimethylchromene (IV) | 3rd | 35 μg |
| Eupatoriochromene (III) | 2nd | [b] |
| | 3rd | [c] |

[a] Assay period 8–10 days, sufficient time for control insects to undergo two molts.
[b] No effect observed to 100 μg.
[c] No effect observed to 200 μg.

## DISCUSSION

Encecalin (II) and eupatoriochromene (desmethylencecalin) (III) have previously been reported as constituents of *H. fitchii* (Bohlmann et al., 1981). 6-Vinyl-7-methoxy-2,2-dimethylchromene (IV), desmethoxyencecalin (V), and 1,8-cineole (I) have not previously been reported as constituents of *H. fitchii,* although they have been identified in other members of the Asteraceae (Bohlmann and Grenz, 1977; Bohlmann and Jakupovic, 1978; Steelink and Marshall, 1979; Bohlmann et al., 1981, 1982, 1983; Proksch and Rodriguez, 1982, 1983). However, to our knowledge, this is the first report of the facile isolation of these chromenes as constituents of a volatile oil.

Chromene derivatives isolated from other members of the Asteraceae have previously been shown to cause various effects in insects (Bowers et al., 1976; Bowers, 1982a,b; Wisdom and Rodriguez, 1982; Proksch and Rodriguez, 1983; Proksch et al., 1983; Rodriguez, 1983; Wisdom et al., 1983). The most biologically active of these chromenes are the precocenes, 6,7-dimethoxy-2,2-dimethylchromene (or ageratochromene) and 7-methoxy-2,2-dimethylchromene. The precocenes have a number of biological effects against insects, including chemosterilant and antijuvenile hormone activities (Bowers, 1981). For example, the precocenes have been shown to induce precocious metamorphosis in *Oncopeltus* nymphs through specific cytotoxic destruction of the parenchymal cells of the corpus allatum (the gland which secretes juvenile hormone) (Bowers et al., 1982). However, we tested the *Hemizonia* chromenes on *Oncopeltus* for antijuvenile hormone activity but did not observe any precocious metamorphosis. In addition, Cupp et al. (1977) reported preimaginal developmental effects of one of the precocenes (ageratochromene) on *Aedes aegypti,* including inhibited pupation and adult emergence. We did not, however, observe any developmental effects by the *Hemizonia* chromenes on *Culex* larvae. Therefore, the

presence of a vinyl or a methylketone moiety, such as found in the *Hemizonia* chromenes, rather than a methoxy substituent, such as found in the precocenes, results in a loss of antijuvenile hormone activity. This conclusion is similar to that of Rodriguez (1983) and coworkers (Proksch et al., 1983), who found moderate insecticidal activity, but no antijuvenile hormone activity with encecalin or eupatoriochromene isolated from *Encelia* species. In fact, Bowers (1982a,b) found that alkoxy substitution of the chromene aromatic ring in the 6th and especially the 7th positions was necessary for antijuvenile hormone activity.

We found the *Hemizonia* chromenes [especially 6-vinyl-7-methoxy-2,2-dimethylchromene (IV)] to be moderately toxic to the *Culex* mosquito larvae, although no antijuvenile hormone activity was observed. These chromenes are therefore probably at least partially responsible for the observed suppressant effect of *H. fitchii* on the mosquito populations in the California ponds. The role of these compounds as defense chemicals in host-plant resistance thus seems apparent. However, in light of the organosoluble nature and expected low water-solubility of the chromenes, other compounds from *Hemizonia* may also be found to contribute to the suppressant effects on mosquito populations. For example, although we found some of the volatile constituents of *H. fitchii,* including 1,8-cineole, and isovaleric, isobutyric, and 2-methylbutyric acids, to be inactive as mosquito larvicides, they may possibly act as repellents to the *Culex* adults. Although we have not yet tested for this possibility, certain unsaturated fatty acids have been reported as ovipositional repellents against *Culex quinquefasciatus* (Hwang et al., 1983). In addition, 1,8-cineole has been reported to repel American cockroach adults (Verma and Meloan, 1981; Maugh, 1982; Scriven and Meloan, 1984), and other compounds isolated from *Hemizonia,* such as acetylenes (Bohlmann et al., 1973) have been isolated from other sources with effects on insects (Jermy et al., 1980). Furthermore, 1,8-cineole may also play an ecologically significant role as an allelopathic substance, since it is known to be a very effective phytotoxin (Muller and Chou, 1972).

Although assays with other insects should be conducted, it does not seem from an economic standpoint that the *Hemizonia* chromenes themselves are of sufficient potency to warrant adaptation into pest management strategies. However, the relative ease of extraction of the *Hemizonia* chromenes, coupled with the availability of *Hemizonia* plant material, make these compounds useful as models for new semisynthetic insecticides. That slight structural differences greatly affect the activity of the chromenes is evident both by comparison of the activities of the precocenes with the *Hemizonia* chromenes, and by comparison of the activities of the *Hemizonia* chromenes among themselves. For example, eupatoriochromene (desmethylencecalin) (III) is structurally very similar to encecalin (II) but is less polar (as shown by its higher $R_f$ value) and much less active against insects than II. The nonpolar character of III is due to the internally hydrogen-bonded hydroxy group (Figure 1, VI), which may also be less

likely to give rise to the reactive epoxide intermediate which is the cytotoxic agent responsible for antijuvenile hormone activity (Brooks et al., 1979; Jennings and Ottridge, 1979; Pratt et al., 1980; Bowers et al., 1982). A similar argument has been proposed to explain the weaker feeding deterrent activity of encecalin (II) as compared to the precocenes (Wisdom et al., 1983). It has also been proposed that chromenes exhibiting free hydroxy groups (such as III) could be more rapidly detoxified (presumably by conjugation and elimination) than compounds bearing methoxy groups (such as the precocenes and II) (Proksch et al., 1983). Thus, semisyntheses utilizing the *Hemizonia* chromenes as starting materials might take advantage of slight structural modifications to enhance insecticidal activity to an economically feasible level.

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# CHEMICAL DEFENSE OF A DORID NUDIBRANCH, *Glossodoris quadricolor*, FROM THE RED SEA

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**Abstract**—The nudibranch, *Glossodoris quadricolor* (Doridacea) feeds on the red sponge *Latrunculia magnifica*, which grows in the reefs of the Gulf of Aqaba, Red Sea. The ichthyotoxic substance from the sponge, latrunculin B, was also indentified in the mucous secretion of the mollusk by TLC, indicating the use of this substance as defense allomone.

**Key Words**—Doridacea, chemical defense, nudibranch, sponge, diet-derived toxin, ichthyotoxin, latrunculin B, *Glossodoris quadricolor*, *Latrunculia magnifica*.

## INTRODUCTION

Nudibranch mollusks lacking a protective shell have developed special defensive mechanisms to escape potential predators. This includes the secretion of strong acids (Thompson, 1960) or other noxious substances that may act as feeding inhibitors or toxic agents towards fishes or other marine animals. A number of studies have demonstrated that nudibranchs use metabolites as defense allomones, which they obtain from their diet, mostly sponges (for reviews see Schulte and Scheuer, 1982; Faulkner and Ghiselin, 1983). *Latrunculia magnifica* is a red-colored branching sponge that grows exposed on the reefs of the Gulf of Aqaba (northern Red Sea). Neeman et al. (1975) had observed that, when squeezed, the sponge exudes a reddish fluid which causes escape reactions in fishes. Toxins named latrunculin A and B were isolated from sponge extracts and structurally characterized as 2-thiazolidinone macrolides, which exhibit marked ichthyotoxic properties (Kashman et al., 1980, 1982) (Figure 1).

The small dorid nudibranch *Glossodoris quadricolor* is found exclusively on the branches of this sponge. With its bright colors of yellow, white, and dark



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FIG. 1. Structures of latrunculins.

blue stripes contrasting with the sponge's red surface, the sea slug is easily seen from a distance of several meters. However, during numerous underwater studies, it was never observed that the nudibranch was attacked by a fish.

This paper describes the analysis of the mucous secretion of the nudibranch, confirming its defensive properties.

## METHODS AND MATERIALS

*Glossodoris quadricolor* and sponge samples of *Latrunculia magnifica* were collected in February 1983 and 1984 in the fringing reefs of the Gulf of Aqaba along the coast of Jordan at a depth of 5–30 m. Samples of five nudibranchs were placed in a small flask containing 5 ml distilled water and slightly agitated; the mucous secretion was decanted and frozen. Nudibranch and sponge samples were kept at −20°C.

Nudibranch and sponge samples were extracted with hexane for 12 hr. and the extracts were evaporated to dryness in a water bath (60°C). These and the aqueous mucous extracts were directly applied onto a thin-layer plate (10 × 10 cm, HPTLC plate, Merck, Darmstadt). Chromatography was performed using the solvent system of benzene–ethylacetate (1:1) according to Neeman et al. (1975). Latrunculin B, kindly provided by Dr. Y. Kashman, Tel-Aviv University, Israel, was used as reference substance. Visualization of the fractions was achieved by spraying the plates with a 0.5% aqueous solution of 2′,7′-dichlorofluorescein or by UV light detection (254 nm). The fractions were marked, scratched from the plate, and eluted from the silica using EtOH.

Ichthyotoxicity was tested using killifish (*Poecilia reticulata*) of uniform size (1.5 cm) placed in beakers containing 10 ml tap water. Various concentrations of aqueous or ethanolic extracts were added; the observation time was 4

hr. Dead fish were removed and replaced by other fish to confirm the results obtained.

## RESULTS AND DISCUSSION

Dissection of the nudibranch *Glossodoris quadricolor* revealed that their pharynx and stomach contain small pieces (up to 1.5 mm diameter) of the red sponge *Latrunculia magnifica*. This confirms the assumption that the mollusk feeds on the sponge. Latrunculin A and B have been isolated from different sponge samples by Kashman et al. (1980). By thin-layer chromatography, only one major and a closely associated minor fraction were detected in aqueous extracts of the nudibranch's mucous secretion; they were identical in $R_f$ value as well as chromatographic separation to the pure latrunculin B reference substance. This component was also present in sponge as well as whole nudibranch extracts, but both extracts contained additional constituents of red, yellow, and blue color, probably the pigments of the sponge (Figure 2).

The pure latrunculin B exhibited marked ichthyotoxicity at a concentration of about 1 mg/liter. The test fish were killed within minutes, confirming earlier observations of Neeman et al. (1975), who determined the $LD_{50}$ of 0.4 mg/liter

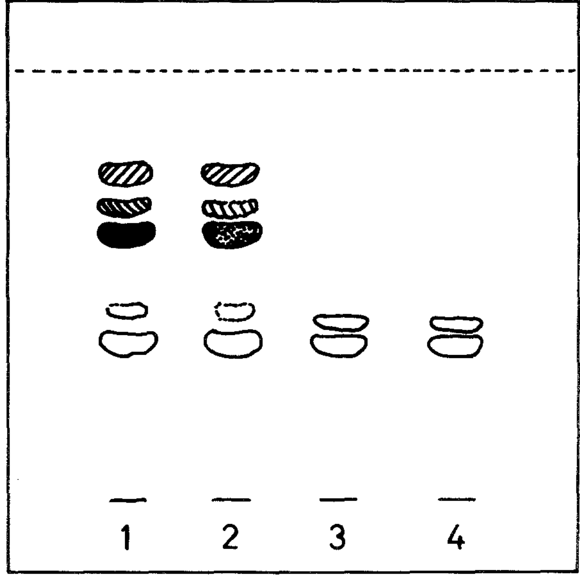


FIG. 2. Thin-layer chromatography of sponge extract (1), nudibranch extract (2), the mucous secretion of the nudibranch (3), and latrunculine B (4). Solvent system: benzene–ethylacetate 1:1, detection UV.

for the toxin fraction. The fishes show excitation, lose their balance in the water, and die rapidly. Extracts from 0.1 g sponge or one nudibranch dissolved in 0.5 ml EtOH were toxic at greater than 10,000-fold dilutions. Eluates of the fractions separated by TLC produced toxic effects to the test fish only when fractions corresponding to latrunculin B were applied. Quantitation of these results was not achieved; however, even dilutions of the eluates between 1:1000 and 1:5000 of the mucus of one nudibranch still produced toxic symptoms.

The results of TLC indicate that the active components of the sponge, the latrunculins, are sequestered by the mollusk in the mucous secretion and are still in an active form as far as toxicity to fish is concerned. Furthermore, comparison by TLC of the sponge extracts and latrunculin B with the substance in the mucus also suggests that latrunculin B is not chemically altered or metabolized by the nudibranch.

It is well known that among marine mollusks, members of the suborder Doridacea feed mainly on sponges and store metabolites from their diet in the dorsum (Schulte and Scheuer, 1982; Faulkner and Ghiselin, 1983). These noxious components provide the protection against predators that they certainly need since they are slow moving and noncryptic animals. The bright color of many reef nudibranchs may also provide a signal to potential predators that may have learned to recognize this particularly unpalatable prey.

This chemical interdependence of a Red Sea nudibranch and a sponge is another example of a prey-predator interrelationship and of the peculiar defense mechanism of this mollusk group.

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# IDENTIFICATION OF VOLATILE SEX PHEROMONE COMPONENTS RELEASED BY THE SOUTHERN ARMYWORM, *Spodoptera eridania* (CRAMER)

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**Abstract**—Analysis of sex pheromone gland extracts and volatile pheromone components collected from the calling female southern armyworm, *Spodoptera eridania* (Cramer), by high-resolution capillary gas chromatography and mass spectroscopy indicated that a number of 14-carbon mono- and diunsaturated acetates and a monounsaturated 16-carbon acetate were produced. Gland extracts also indicated the presence of (Z)-9-tetradecen-1-ol. However, this compound was not found in collections of volatiles. Field trapping studies indicated that the volatile blend composed of (Z)-9-tetradecen-1-ol acetate (60%), (Z)-9-(E)-12-tetradecadien-1-ol acetate (17%), (Z)-9-(Z)-12-tetradecadien-1-ol acetate (15%), (Z)-9-(E)-11-tetradecadien-1-ol acetate (5%), and (Z)-11-hexadecen-1-ol acetate (3%) was an effective trap bait for males of this species. The addition of (Z)-9-tetradecen-1-ol to the acetate blends tested resulted in the capture of beet armyworm, *S. exigua* (Hubner), males which provides further evidence that the alcohol is a pheromone component of this species.

**Key Words**—*Spodoptera eridania*, southern armyworm, *Spodoptera exigua*, beet armyworm, Lepidoptera, Nocteridae, pheromone, attractant, (*Z*)-9-tetradecen-1-ol acetate, (*Z*)-9-(*E*)-12-tetradecadien-1-ol acetate, (*Z*)-9-(*E*)-12-tetradecadien-1-ol acetate, (*Z*)-9-(*E*)-11-tetradecadien-1-ol acetate, (*Z*)-11-hexadecen-1-ol acetate, (*Z*)-9-tetradecen-1-ol, behavior.

## INTRODUCTION

Members of the genus *Spodoptera* are distributed throughout the world and include a number of species that cause substantial damage to agricultural crops. The southern armyworm, *Spodoptera eridania* (Cramer), is one of three major Northern American pests in this genus and is present in much of Florida throughout the year. Larvae are general feeders and cause damage to a wide variety of commercial vegetable crops (Metcalf et al., 1962). Larvae have also recently been reported as a pest of sunflower (Mitchell, 1984).

Efforts to identify the sex pheromone of the southern armyworm (SAW) by Jacobson et al. (1970) led to the identification of a 5:1 blend of (Z)-9-tetradecen-1-ol acetate (Z9–14:AC) and (Z)-9-(*E*)-12-tetradecadien-1-ol acetate (*Z*9,*E*12–14:AC) from methylene chloride extracts of the excised abdomens of virgin SAW females. Although *Z*9 – 14:AC stimulated males in laboratory bioassays, no behavioral function was defined for *Z*9,*E*12–14:AC. Field tests using the individual compounds, a 1:1 blend of the two, and methylene chloride extracts of female abdomens indicated that neither the individual compounds nor the binary mixture were as attractive as the methylene chloride extracts (Redfern et al., 1971). Subsequent field tests using the individual compounds and 11 different blends of the two indicated that none of the synthetic lures were more attractive than blank traps (Mitchell and Doolittle, 1976). This present study reports the identification of a blend of sex pheromone components collected from volatiles released by calling SAW females, the identification of pheromone components in gland extracts, and the results of field trapping studies used to assess the effectiveness of various blends of synthetic compounds for SAW males.

## METHODS AND MATERIALS

*General.* The SAW females used in this study were collected as larvae in the field and maintained on an artificial diet in the laboratory (Burton, 1970). After pupation the insects were separated by sex and the females placed in 3.8-liter paper cartons with wire screen tops. Pupae and adults were maintained at 25°C, relative humidity of 70%, and in a reversed 16:8 hr light–dark cycle. Newly emerged females were collected daily and placed in 30 × 30 × 30-cm Plexiglas sleeved cages. Adults were provided with a 10% sucrose solution on cotton balls as a food source.

*Pheromone Collection.* Extracts of pheromone glands were prepared by excising the thermal three abdominal segments from calling females 2–5 days old and either soaking the tissue for 30 min in a conical microvial containing 5 $\mu$l of isooctane (Fisher, 99 mol/100 ml) or dipping them in the solvent for ca. 10 sec. Volatile components released by calling females were collected by entrainment on charcoal microcollection filters (Grob and Zurcher, 1976) by push-

ing purified air at 500 ml/min over individuals held in chambers similar to that described by Tumlinson et al. (1982). Volatiles were recovered from the filters using a previously described technique (Tumlinson et al., 1982) slightly modified to include rinsing the walls of the receiving vessel with 15 $\mu$l of redistilled pentane after extracting the filters and the addition of appropriate internal standards prior to the addition of 10 $\mu$l of isooctane and volume reduction under $N_2$.

*Chemical Analysis.* Capillary gas chromatographic (GC) analyses of all natural product samples and synthetic standards were performed using either a Hewlett-Packard 5710® or Varian 3700® GC equipped with splitless injectors and flame ionization detectors. Helium was used as the carrier gas in both instruments at a linear flow of velocity of 18 cm/sec. Capillary GC columns used for analysis included a 35-m × 0.15-mm (ID) OV-1 column, a 45-m × 0.3-m (ID) column coated with SP2340® (Supelco), and a 60-m × 0.25-mm (ID) fused silica column coated with SP2330® (Supelco). Samples were injected at an initial temperature of 80° in the splitless mode (injector purge at 60 sec). Oven temperatures were programed after 2 min at 30°/min to final temperatures of 180°C (OV-1), 130°C (SP2340), and 170°C (SP2330). Chromatographic data were collected and analyzed using a Perkin Elmer CIT II® system and 3600 data station. Retention times of both natural products and synthetic standards were converted to equivalent chain length (ECL) units with slight modification (Jamison and Reid, 1969) using the acetates of saturated primary alcohols as the function retention index (Swoboda, 1962) regardless of the compounds functionality.

Mass spectra were obtained with a Finnigan model 3200® chemical ionization mass spectrometer equipped with a capillary GC inlet and splitless injector. Both the OV-1 and SP2340 columns used in GC analysis were used in the GC-mass spectrometer. The total effluent from the columns was introduced directly into the ionization source. Samples were injected at 80°C, and the injector was purged at 60 sec at which time the GC was temperature programed at 20°C/min to final temperatures of 180°C (SP2340) and 220°C (OV-1). Helium, at a linear flow velocity of 27 cm/sec, was used as the carrier gas and methane was used as the reagent gas.

*Synthetic Chemicals and Blend Formulation.* The following synthetic chemicals were obtained from Albany International, Chemicals Division: Z9–14:AC, (*Z*)-9-tetradecen-1-ol (Z9–14:OH), (*Z*)-9-(*E*)-12-tetradecadien-1-ol (Z9,*E*12–14:OH), (*Z*)-9-(*E*)-11-tetradecadien-1-ol acetate (Z9,*E*11–14:AC), (*Z*)-11-hexadecen-1-ol acetate (Z11–16:AC) and (*Z*)-11-hexadecen-1-ol (Z11-16:OH). Z9,*E*12–14:AC, and (*Z*)-9-(*Z*)-12-tetradecadien-1-ol acetate (Z9,*Z*12–14:AC) were obtained from Zoecon Coprporation. All synthetic compounds were analyzed by capillary GC using the OV-1 and SP2330 columns and were purified when necessary by high-performance liquid chromatography on 25 × 2.5-cm (OD) $AgNO_3$–silica column eluted with toluene. Synthetic compounds used in blend formulations were assessed as being >90% pure.

The A.H. Thomas rubber septa (8753-D22) used as dispensers for all field studies were Soxhlet extracted for 48 hr in methylene chloride and air dried prior to use. Blends were formulated to release the desired ratios of compounds in the blend. Volatiles released from the septa were collected on the microcollection filters and analyzed by capillary GC to ensure that the desired ratios were being released prior to using the septa in the field. All septa were loaded with 200 $\mu$g of each test blend.

*Field Studies.* Field studies were conducted in sunflower fields in Alachua County, Florida during September–December 1982. Pherocon 1C® sticky traps were positioned 30 m apart at crop height throughout the field in a complete randomized block design. Traps were baited with either three virgin females or a rubber septum lure. Females were replaced at three-day intervals while septa were changed after three weeks. Traps in each of the four replicates were randomized daily. Capture data were recorded daily and the data transformed $\sqrt{x + 0.5}$ prior to analysis of variance and Duncan's multiple-range test.

## RESULTS

*Isolation and Identification.* GC analysis of extracts obtained from calling SAW females indicated that the two major components of the extract had ECL units corresponding to *Z*9–14:AC and *Z*9,*E*12–14:AC on all three GC columns (peaks 3 and 4; Figure 1A). As indicated in Figure 1, numerous other peaks were also present with regularity. Peaks 1, 2, and 7 had ECL units coincident with *Z*9–14:OH, *Z*9,*E*12–14:OH, and *Z*11–16:OH on both the OV-1 and SP2340 columns. These peaks were not present in chromatograms of extracts injected onto the SP2330 column. However, the SP2330 column did not chromatograph alcohol standards which supported the above assignments for these peaks. The retention characteristics of peak 5 on all three columns suggested that it was a geometric isomer of *Z*9,*E*12–14:AC and it had ECL values identical to *Z*9,*Z*12–14:AC. Peak 6 ECL values of 1405.6 (OV-1), 1558.2 (SP2340), and 1697.2 (SP2330). No tentative assignment was made for this compound prior to mass spectrometry. Peaks 8 and 9 had ECL values coincident with *Z*9–16:AC and *Z*11–16:AC on all three columns.

Mass spectrometry confirmed that peaks 1, 3, 4, 5, and 9 in Figure 1 were in fact a monounsaturated $C_{14}$ alcohol, a monounsaturated and two diunsaturated $C_{14}$ acetates, and a monounsaturated $C_{16}$ acetate. No spectra were obtained for peaks 2, 7, and 8 because of insufficient quantities, and therefore the presence of *Z*9,*E*12–14:OH, *Z*11–16:OH and *Z*9–16:AC in the extracts cannot be confirmed. The mass spectrum of peak 6 (Figure 1A) indicated a mass of 252 and major fragments at 61, 89, 191, and 193, all of which are indicative of diunsaturated $C_{14}$ acetate.

A review of the literature revealed that *Z*9,*E*11–14:AC had been identified

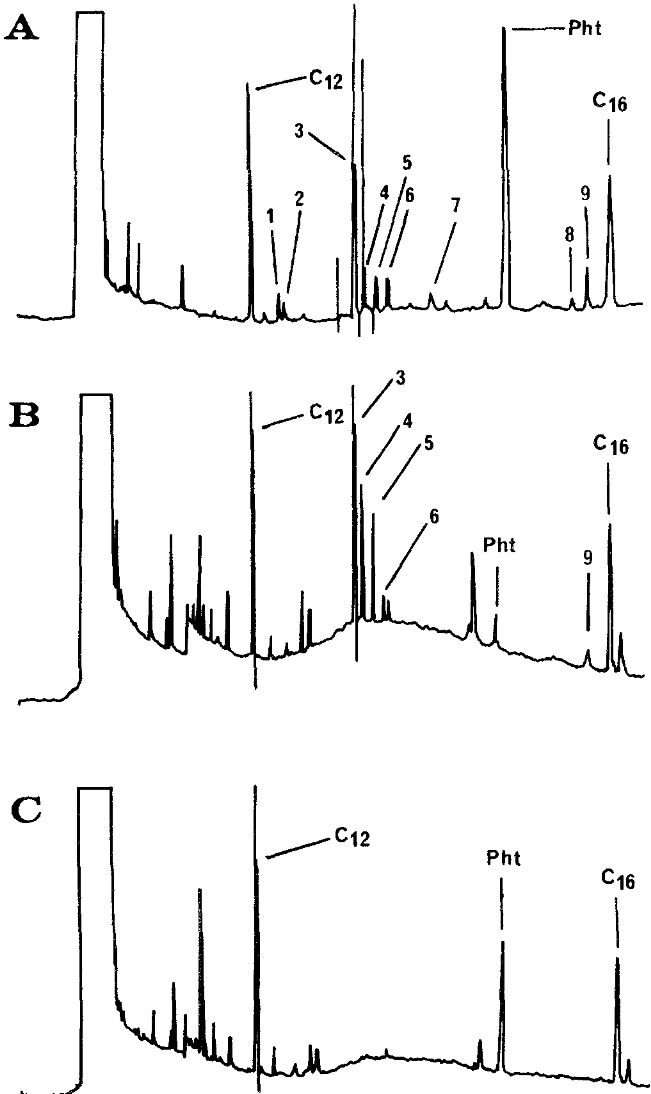


FIG. 1. GC chromatograms on a 35-m × 0.15-mm (ID) glass column coated with OV-1. (A) Peaks eluting from the injection of 10 female equivalents of the ovipositor dip obtained from calling females. (B) Peaks eluting during the analysis of volatiles released by four individually caged females over a 1-hr period. (C) Peaks eluting from the analysis of four system blanks during the same 1-hr period as B. $C_{12}$ = dodecan-1-ol acetate (internal standard), $C_{16}$ = hexadecan-1-ol acetate (internal standard), 1 = *Z*9–14:OH, 2 = *Z*9,*E*12–14:OH, 3 = *Z*9–14:AC, 4 = *Z*9,*E*12–14:AC, 5 = *Z*9,*Z*12–14:AC, 6 = *Z*9,*E*11–14:AC, 7 = *Z*11–16:OH, 8 = *Z*9–16:AC, 9 = *Z*11–16:AC. Pth = phthalate.

TABLE 1. CHEMICAL COMPONENTS IDENTIFIED FROM OVIPOSITOR WASHES AND VOLATILE COLLECTIONS FROM CALLING SAW FEMALES

| | Mean percentages of each compound[a] | | |
|---|---|---|---|
| Compound | Gland extract (ng of total blend/female) | Volatile (ng of total blend/female/min) | Loaded onto septa (μg of total blend/lure) |
| Z9-14:OH | 3.8 | 0 | 0 |
| Z9-14:AC | 57.2 | 60.6 | 47.5 |
| Z9,E12-14:AC | 23.1 | 16.9 | 18.1 |
| Z9,Z12-14:AC | 3.0 | 15.2 | 15.2 |
| Z9,E11-14:AC | 4.9 | 4.6 | 6.8 |
| Z11-16:AC | 8.0 | 2.7 | 12.4 |
| | 3.5 | 0.09 | 200 |

[a]Based on 10 chromatograms using both the OV-1 and SP2340 columns for both the extracts and volatile collections.

from gland extracts of other *Spodoptera* species (Nesbitt et al., 1973; Tamaki et al., 1973; Tamaki and Yushima, 1974; Campion, 1975). Chromatography of this compound indicated that it had ECL values similar to peak 6 on all three GC columns. Cochromatography of extracts with 5 ng of each of the six tentatively identified compounds on all three GC columns added further support to the chemical assignments. The percentages of these compounds in ovipositor extracts are shown in Table 1.

GC and mass spectral analyses of the volatile blends released by calling females (Figure 1B) indicated that Z9-14:AC, Z9,E12-14:AC, Z9,Z12-14:AC, Z9,E11-14:AC, and Z11-16:AC were present in the ratio indicated in Table 1. No Z9-14:OH, Z9,E12-14:OH, Z11-16:OH, or Z9-16:AC was detected in any of the volatile samples. However, this does not necessarily indicate that these compounds are not released in minute amounts, nor does it preclude the possibility that other unidentified compounds are released.

*Field Studies.* Results of field trapping studies are summarized in Table 2. Although a few SAW males were captured in traps baited with the binary mixture of Z9-14:AC and Z9,E12-14:AC, this blend was found to be the least effective of those tested. The addition of Z11-16:AC to the binary mixture significantly increased the number of males captured; however this blend did not capture as many males as did female-baited traps. No increase in the number of males captured was evident when Z9,Z12-14:AC was included in the blend. However, this does not imply that this compound is without a behavioral function. As indicated in Table 2, the five-compound volatile blend which included

TABLE 2. COMPARISON OF STICKY TRAP CAPTURES OF SAW AND BAW MALES USING DIFFERENT BLENDS OF VOLATILE PHEROMONE COMPONENTS OF SAW FEMALES AND 3.8% Z9-14:OH[a]

| 3 Females | Z9-14:OH | Z9-14:AC | Z9,E12-14:AC | Z9,Z12-14:AC | Z9,E11-14:AC | Z11-16:AC | Mean no. of males/night | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | SAW | BAW |
| + | | | | | | | 10.5 b | 0.0 f |
| | | + | + | | | | 3.5 c | 0.0 f |
| | | + | + | | | + | 8.0 b | 0.0 f |
| | | + | + | + | | + | 8.1 b | 0.4 f |
| | | + | + | + | + | + | 14.3 a | 0.0 f |
| | + | + | + | | | + | 7.1 b | 4.5 g |
| | + | + | + | + | | + | 7.9 b | 2.0 h |

[a]Means followed by the same letter are not significantly different in a Duncan's multiple-range test at $P = 95\%$. Four replicates over four weeks. A + indicates the presence of the compound indicated in the test blend.

*Z*9,*E*11-14:AC was the most effective lure tested and captured significantly greater numbers of males than did female-baited traps.

Table 2 also shows the mean number of beet armyworm (BAW) [*Spodoptera exigua* (Hubner)], males captured in each of the traps used in the above study. As indicated, blends that did not contain Z9-14:OH did not capture BAW males, nor did SAW females. The addition of Z9-14:OH to the three-component blend of Z9-14:AC, Z9,E12-14:AC, and Z11-164:AC resulted in the capture of the largest number of BAW males, and the addition of Z9,Z12-14:AC to this blend resulted in a significant reduction in the number of BAW males captured.

## DISCUSSION

The use of 14-carbon mono- and diunsaturated acetates as major components of the SAW pheromone follows the general trend within the genus, and various combinations of these components have been identified from all of the *Spodoptera* species studied to date (Beevor et al., 1975; Nesbitt et al., 1973; Tamaki and Yushima, 1974; Tamaki et al. 1973, 1978; Tumlinson et al., 1981, 1983). Acetates of primary alcohols having either longer or shorter carbon skeletons have only been identified from North American species (Tumlinson et al., 1983). Further study may indicate the presence of $C_{12}$ and/or $C_{16}$ acetates in other cosmopolitan species. Of the five compounds identified from SAW females, only Z9,Z12-14:AC did not appear to affect trap capture significantly.

However, assessments of the behavioral functions of individual components based on trap capture data is vague, and therefore, Z9,Z12–14:AC may play a significant role in courtship (see Baker and Cardé, 1979). Obviously, more critical assessments of the functions of individual components are necessary.

An interesting aspect of this study was the identification of Z9–14:OH in the gland extracts but not in the volatile pheromone blend. Similar cases have been reported for other Lepidoptera (Weatherston and McLean, 1974; Silk et al., 1980; Teal et al., 1981, 1984) and probably indicate a role in biosynthesis. Tumlinson et al. (1981) demonstrated that Z9,*E*12–14:AC and Z9–14:OH were necessary components of the BAW pheromone and that Z9,*E*12–14:OH and Z9–14:AC were present in significant amounts. However, studies by Persoons et al. (1981) indicated that the pheromone blend contained only the acetates. Our field studies support the work of Tumlinson et al. (1981) that indicated that Z9–14:OH is a pheromone component of the BAW. Although Z9–14:OH does not appear to affect the capture of SAW males, it is possible that it plays an important role in the reproductive isolation of SAW males from other species during close-range courtship.

The five-component volatile blend identified in our study is an effective lure for SAW males and is currently being used for population monitoring. However, more behavioral and chemical research is required to assess the behavioral effects of each component and to define more accurately the precise volatile pheromone blend in terms of the minor components produced and ratios of the components released.

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# SEX ATTRACTANTS FOR GEOMETRID AND NOCTUID MOTHS
## Field Trapping and Electroantennographic Responses to Triene Hydrocarbons and Monoepoxydiene Derivatives[1]

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**Abstract**—Male moths belonging to 17 species of Geometridae and nine species of Noctuidae were captured in traps baited with synthetic chemicals as part of a field screening program. The compounds tested were the $C_{18}$–$C_{22}$ homologs of: (1) (3*Z*,6*Z*,9*Z*)-triene hydrocarbons; (2) mixtures containing equal quantities of (3*Z*,6*Z*)-*cis*-9,10-expoxydienes, (3*Z*,92*Z*)-*cis*-6,7-epoxydienes, and (6*Z*,9*Z*)-*cis*-3,4-epoxydienes; (3) (3*Z*,6*Z*)-9*S*,10R-epoxydienes; (4) (3*Z*,6*Z*)-9*R*,10S-epoxydienes; and (5) (3*Z*,6*Z*,9*Z*,11*E*)-nonadecatetraene. Field captures and electroantennographic assays revealed a high degree of specificity in the responses of many species to the synthetic chemicals. In several species the ability of males to discriminate between the 9*S*,10*R* and 9*R*,10*S* enantiomers of the monoepoxydiene isomers was clearly shown. Synergists and inhibitors were discovered for several of the reported attractants, some of which were not previously known to have semiochemical activity. The geometrid moths captured included *Epirrhoe sperryi* (Herbulot), *Mesoleuca ruficillata* (Guenée), *Triphosa haesitata* (Guenée), *Metanema inatomaria* (Guenée), *Prochoerodes transversata* (Drury), *Cabera erythemaria* (Guenée), *Synaxis jubararia* (Hulst), *Dysstroma brunneata ethela* (Hulst), *Eulithes testata* (Linnaeus), *Sicya macularia* (Harris), *Xanthorhoe iduata* (Guenée), *X. abrasaria aquilonaria* (Herrich-Schäffer), *X. munitata* (Hübner), *Itame loricaria* (Eversmann), *Eupithecia annulata* (Hulst), *E. rovocastaliata* (Packard) and *E. satyrata dodata* (Taylor). The noctuid moths captured included *Bleptina caradrinalis* (Guenée), *Idia americalis* (Guenée), *I. aemula* (Hübner), *Rivula propinqualis* (Guenée), *Lomanaltes eductalis* (Walker), *Spargaloma sexpunctata* (Grote), *Caenurgina distincta* (Neumuller), *Euclidia cuspidea* (Hübner), and *Zale duplicata* (Bethune). Six of

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the nine noctuid species captured belong to three subfamilies for which sex attractants had not been reported previously. Details for the stereospecific synthesis of (3*Z*,6*Z*)-*cis*-9,10-epoxydienes are also reported.

**Key Words**—Sex attractants, Lepidoptera, Geometridae, Noctuidae, stereospecific synthesis, (3*Z*,6*Z*,9*Z*)-triene hydrocarbons, (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxydienes, (3*Z*,6*Z*,9*R*,10*S*)-9,10-epoxydienes, electroantennogram.

## INTRODUCTION

Sex pheromones and sex attractants are known for approximately 150 species of North American Noctuidae (Roelofs, 1979; Steck et al., 1982). Although specific sex attractants have been developed for most of the economically important noctuid pests, these represent only a small fraction of the 2925 species in 18 subfamilies which inhabit America north of Mexico (Hodges et al., 1983). Until very recently, noctuid species for which sex pheromones or attractants were known belonged to only nine of these subfamilies (Table 1).

In North America, the Geometridae consists of 1404 species of moths divided into six subfamilies and is second in size to the Noctuidae (Hodges, 1983). Despite the large number of species, sex pheromones have only recently been reported for the winter moth (Roelofs et al., 1982; Bestmann et al., 1982), the giant looper (Becker et al., 1983), and the fall cankerworm (Wong et al., 1984a,b).

The failure of noctuid species from the nine subfamilies listed on the right in Table 1 and geometrid species to respond to our annual field surveys conducted between 1978 and 1981 with over 300 saturated and olefinic, straight-

TABLE 1. NOCTUIDAE SUBFAMILIES[a] AND CHEMICALLY DEFINED SEX ATTRACTANTS

| Subfamilies for which attractants have been reported to 1983 | Subfamilies having no known sex attractants to 1983 |
|---|---|
| Plusiinae (74)[b] | Herminiinae (83) |
| Acontiinae (178) | Rivulinae (16) |
| Pantheinae (23) | Hypenodinae (21) |
| Acronictinae (99) | Hypeninae (49) |
| Amphipyrinae (548) | Catocalinae (391) |
| Cuculliinae (352) | Euteliinae (16) |
| Hadeninae (425) | Sarrothripinae (14) |
| Noctuinae (415) | Nolinae (16) |
| Heliothinae (179) | Agaristinae (26) |

[a]The system used is that of Hodges et al. (1983) and refers only to North American species, north of Mexico.
[b]The number of species within the subfamily is in parentheses.

chain acetates, aldehydes, and alcohols led us to suspect that some of these noctuid and geometrid moths may utilize pheromones whose structures are significantly different from the majority of known lepidopteran pheromones, and stimulated us to perform a study. Our investigation of lepidopteran pheromones and attactants is based on two approaches: (1) identification of the insect-derived pheromone, and (2) field screening of potential attractants. We have successfully utilized the first approach for identification of pheromones for two geometrid species, the fall cankerworm, *Alsophila pometaria* (Harris), and the spring cankerworm, *Paleacrita vernata* (Peck) (Underhill et al., unpublished), and one noctuid species, the forage looper, *Caenurgina erechtea* (Underhill et al., 1983). The second approach, field screening of potential attractants, has also been successful and is the subject of this report. Compounds tested as candidate lures were (3*Z*,6*Z*,9*Z*)-eicosatriene and (3*Z*,6*Z*,9*Z*)-heneicosatriene, pheromone components of *C. erechtea*. Other compounds tested included the $C_{18}$, $C_{19}$, and $C_{22}$ homologs of the above triene hydrocarbons, several monoepoxydiene analogs, and (3*Z*,6*Z*,9*Z*,11*E*)-nonadecatetraene, a pheromone component of the fall cankerworm (Wong et al., 1984a). We also report details of a stereospecific synthesis of (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxyheneicosadiene, the saltmarsh caterpillar pheromone, along with $C_{18}$, $C_{19}$, $C_{20}$, and $C_{22}$ homologs, and a homologous series of 9*R*,10*S* enantiomers.

## METHODS AND MATERIALS

*Synthesis.* General procedures along with chromatographic and analytical instrumentation have been previously described (Wong et al., 1984b). Abbreviations used to describe PMR signals are as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad singlet (bs). Unless otherwise noted, product isolation was accomplished by drying the organic phase derived from product extraction over anhydrous magnesium sulfate followed by filtration and concentration under reduced pressure in a rotary evaporator.

Optical rotations were measured with a Perkin-Elmer model 141 polarimeter on chloroform solutions (C in g/100 ml) contained in a 10-cm path length cell. Determinations of enantiomeric purities of the epoxyalcohol intermediates (Va and Vb) were achieved by PMR observations on the complexes formed between the epoxyalcohols and *tris*-(*d*,*d*-dicampholylmethanato)europium (III) (Alfa Products) or the (+)-$\alpha$-methoxy-$\alpha$-(trifluoromethyl)phenylacetate derivatives of the epoxyalcohols (Dale et al., 1969).

*Unsaturated Hydrocarbons.* The (3*Z*,6*Z*,9*Z*)-triene hydrocarbons ($C_{18}$–$C_{22}$) [(3*Z*,6*Z*,9*Z*)-X:H where $X$ = 18–22] used in the field survey and for electroantennoghraphic (EAG) assays were synthesized from methyl linolenate (NuChek Corp., Elysian, Minnesota) via a copper-catalyzed Grignard coupling as previously described (Underhill et al., 1983). More recently we have pre-

pared these trienes from ethyl linolenate (Sigma, St. Louis, Missouri) by the coupling of (9Z,12Z,15Z)-1-tosyloxy-octadecatriene with an appropriate lithium dialkylcuprate reagent (Conner et al., 1980; Heath et al., 1983). Other unsaturated hydrocarbons used primarily for EAG assays include (6Z,9Z)-diene hydrocarbons ($C_{18}$–$C_{22}$) and (6Z,9Z,12Z)-triene hydrocarbons ($C_{18}$, $C_{20}$, $C_{21}$, and $C_{22}$), and were also prepared by one or both procedures described above from methyl linolenate and methyl-γ-linolenate (NuChek Corp.), respectively. Two tetraenes, (6Z,9Z,12Z,15Z)-20:H and (6Z,9Z,12Z,15Z)-22:H, were prepared by lithium tetrahydroaluminate reductions of the tosylate derivatives of (5Z,8Z,11Z,14Z)-20:OH (arachidonyl alcohol) and (7Z,10Z,13Z,16Z)-22:OH (both from NuChek Corp.). The purities of all unsaturated hydrocarbons were greater than 98% as determined by GC analysis on capillary columns.

*Linolenyl Alcohol, Acetate, and Aldehyde.* These compounds were also used primarily for EAG assays. Linolenyl alcohol and linolenyl acetate (both 99%) were purchased from NuChek Corp. and used without further purification. The aldehyde [(9Z,12Z,15Z)-18:Ald] was prepared by oxidation of linolenyl alcohol with pyridinium chlorochromate (Corey and Suggs, 1975).

*Monoepoxydiene Derivatives of (3Z,6Z,9Z)-Triene Hydrocarbons.* Mixtures comprised of approximately equal quantities of (3Z,6Z)-*cis*-9,10-epoxydienes, (3Z,9Z)-*cis*-6,7-epoxydienes, and (6Z,9Z)-*cis*-3,4-epoxydienes, all from 18 to 22 carbons in length, were prepared by *m*-chloroperbenzoic acid oxidations of the parent hydrocarbons. Details for the preparation of the monoepoxydienes of (3Z,6Z,9Z)-19:H, which shall be referred to as the combined monoepoxides of (3Z,6Z,9Z)-19:H [CME-(3Z,6Z,9Z)-19:H], will be given as a representative example and are as follows: *m*-chloroperbenzoic acid (345 mg, 2 mmol) was added to 524 mg of (3Z,6Z,9Z)-19:H dissolved in 10 ml of dichloromethane. The reaction mixture was stirred at 22°C for 1 hr, diluted with 20 ml dichloromethane, and washed successively with 30-ml portions of 5% aqueous sodium bicarbonate and saturated aqueous sodium chloride. Product isolation afforded 530 mg of crude product composed of unreacted (3Z,6Z,9Z)-19:H (22.4%), CME-(3Z,6Z,9Z)-19:H (57.6)%, and overoxidized products (20.0%). Isolation of the CME-(3Z,6Z,9Z)-19:H was accomplished by flash chromatography on a 20-cm × 3.5-cm-ID column of Kieselgel 60 (20-ml fractions). Elution with hexane followed by hexane–ether (95:5, v/v) afforded a fraction containing 259 mg of a colorless oil which revealed three partially separated peaks accounting for 99% of the sample on GC analysis. Ions at 279 (M + 1) and 261 [(M + 1) − 18] were found in the chemical ionization mass spectra of all three peaks which were completely resolved by the 60-m × 0.32-mm-ID DB-5 column used in the GC-MS analysis. These ions and other characteristic ions that were present were consistent with the molecular weight and structures of the desired monoepoxydiene isomers [(3Z,6Z)-*cis*-9,10-epoxy-19:H, (3Z,9Z)-*cis*-6,7-epoxy-19:H, and (6Z,9Z)-*cis*-3,4-epoxy-19:H]. Signals in the 90-MHz [$^1$H]NMR spectrum of the product mixture were also consistent

with the desired structures: δ 5.7–5.1 (4H, m, vinyl), 3.0–2.7 (3.3H, m, bis-allylic and epoxide), 2.4–1.9 (5.3H, m, allylic), 1.1 (14H, bs, methylene), 0.95–0.75 (6H, 2t, methyl).

The combined monoepoxides of (3*Z*,6*Z*,9*Z*)-18:H, -20:H, -21:H, and -22:H were prepared by the procedure described above. Satisfactory spectroscopic analyses and purities of 99% were obtained for all four product mixtures.

*2,5-Octadiyn-1-ol(I).* 1-Tetrahydropyranyloxy-2-propyne (56 g, 400 mmol) was prepared from 2-propyn-1-ol by the procedure of Parham and Anderson (1948) and added to a freshly prepared solution of ethylmagnesium bromide in THF (440 mmol). After 1 hr at 5°C, the Grignard reagent was treated with 2.3 g (8 mmol) of cuprous bromide (98%, Aldrich) followed, after 0.5 hr, by the slow addition of 1-tosyloxy-2-pentyne [95.2 g, 400 mmol; prepared from 2-pentyn-1-ol (Farchan) by the method of Sendega et al. (1968)]. The reaction mixture was then warmed to 21°C, stirred for 5 hr, and quenched with 400 ml of saturated aqueous ammonium chloride. Ether extraction followed by product isolation afforded crude 1-tetrahydropyranyloxy-2,5-octadiyne which was treated with 300 ml of 2.5% *p*-toluenesulfonic acid (*p*-TsOH) in methanol for 2 hr at 21°C to effect hydrolysis of the protecting group. The crude product obtained after standard work-up procedure was distilled through a 10-cm Vigreux column to afford 39.2 g of pure 2,5-octadiyn-1-ol (80.4% yield); bp 72–75°C (0.5 torr). [$^1$H]NMR (90 MHz, $CDCl_3$):δ 4.23 (2H, t, $J$ = 2.1 Hz, $-C\underline{H}_2-OH$), 3.17 (2H, quintet, $J$ = 2.2 Hz, $-C{\equiv}C-C\underline{H}_2-C{\equiv}C-$), 2.4–2.0 (3H, m, $CH_3-C\underline{H}_2-$ and $-O\underline{H}$), 1.10 (3H, t, $J$ = 7.3 Hz, $C\underline{H}_3-$).

*1-Tosyloxy-2,5-octadiyne (II).* Freshly powdered potassium hydroxide (42 g) was added over 0.5 hr to an ether solution of I (36.6 g, 300 mmol) and *p*-toluenesulfonyl chloride (*p*-TsCl) maintained at −20°C. The reaction mixture, which turned dark brown on addition of potassium hydroxide, was stirred at −20°C for 2 hr, warmed to −10°C and stirred for an additional 2-hr period. Water (30 ml) was added to the reaction and the ether phase separated. The aqueous layer was extracted with ether (2 × 200 ml) and the combined ether extracts worked up in the usual manner to give the dark brown crude tosylate. Partial purification was achieved by elution of the crude product through a column (20 cm × 4.5 cm ID) of Kieselgel 60 with 1.6 liter of hexane–ethyl acetate (85:15, v/v). Solvent removal left 76.2 g (92.0% yield) of 1-tosyloxy-2,5-octadiyne which GC analysis revealed to be 76% pure: [$^1$H]NMR (90 MHz, $CDCl_3$): δ 7.80 (2H, d, $J$ = 9.0 Hz, aromatic), 7.30 (2H, d, $J$ = 9.0 Hz, aromatic), 4.67 (2H, t, $J$ = 2.2 Hz, $-CH_2-O-Ts$), 3.0 (2H, quintet, $J$ = 2.2 Hz, $-C{\equiv}C-C\underline{H}_2-C{\equiv}C-$), 2.4 (3H, s, $C\underline{H}_3-C_6H_4-$), 2.1 (2H, tq, $J$ = 2.2 Hz and 7.5 Hz, $CH_3-C\underline{H}_2-$), 1.07 (3H, t, $J$ = 7.5 Hz, $C\underline{H}_3-CH_2-$).

*1-*tert*-Butyldimethylsilyloxy-2,5,8-undecatriyne (III).* A THF solution of 1-*tert*-butyldimethylsilyloxy-2-propyne (34.0 g, 200 mmol), prepared by standard procedure from 2-propyn-1-ol (Corey and Venkateswarlu, 1972), was cooled to 4°C and treated with 100 ml of a 2 M solution of ethylmagnesium bromide in

THF (Aldrich). The resultant mixture was stirred for 1 hr and treated with 1.14 g (4 mmol) of cuprous bromide. After another hour, a solution of 1-tosyloxy-2,5-octadiyne (72.6 g of 76% purity, ~ 200 mmol) in 50 ml THF was added dropwise. The reaction mixture was warmed to 21°C, stirred for 3 hr, and quenched with 400 ml of saturated aqueous ammonium chloride. Extraction with ether (3 × 250 ml) followed by product isolation afforded the crude triyne. Vacuum distillation through a 10-cm Vigreux column gave 34.8 g of 1-t-butyldimethylsilyloxy-2,5,8-undecatriyne (63.5% yield); bp 126–128°C (0.3 torr). GC analysis revealed a purity of 93.5%. [$^1$H]NMR (90 MHz, $CDCl_3$): δ 4.23 (2H, t, $J$ = 2.2Hz, $-C\underline{H}_2-O$), 3.08 (4H, m, $-C{\equiv}C-C\underline{H}_2-C{\equiv}C-$), 2.10 (2H, tq, $J$ = 2.2 Hz and 7.5 Hz, $CH_3C\underline{H}_2-$), 1.10 (3H, t, $J$ = 7.5Hz, $C\underline{H}_3-$),

$$0.90\left[9H,\ s, -\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{\underset{|}{\overset{|}{Si}}}}-C(C\underline{H}_3)_3\right],\ 0.10\left[6H,\ s,\ -\underset{\displaystyle C\underline{H}_3}{\overset{\displaystyle C\underline{H}_3}{\underset{|}{\overset{|}{Si}}}}-C(CH_3)_3\right].$$

*(2Z,5Z,8Z)-Undecatrien-1-ol (IV).* Borane–dimethylsulfide complex (24 ml of 10 M liquid, Aldrich) was added to a vigorously stirred solution of dry cyclohexene (39.4 g, 480 mmol) dissolved in 175 ml of dry THF and maintained below 10°C. After 1 hr, 10.96 g (40 mmol) of III was added to the white precipitate of dicyclohexylborane. The resultant mixture was warmed to 21°C, stirred for 20 hr, and treated with 55 ml of glacial acetic acid for 4 hr at 60°C. Then the mixture was cooled in an ice bath, made basic with 200 ml of 6 N sodium hydroxide and cautiously treated with 57 ml of 30% hydrogen peroxide. Ether extraction (3 × 100 ml) followed by product isolation afforded the crude (2Z,5Z,8Z)-1-*t*-butyldimethylsilyloxy-2,5,8-undecatriene together with cyclohexanol. This crude product was combined with the crude product from a second experiment performed on 30 mmol of III and treated with 100 ml of 1 M tetrabutylammonium fluoride in THF (Aldrich) for 15 hr at 21°C. The product obtained after aqueous work-up and ether extraction was partially purified by vacuum distillation (bp 80–83°, 0.4 torr) to remove the cyclohexanol. Flash chromatography of the distillate (25-cm × 4.0-cm-ID column; hexane–ethyl-acetate, 4 : 1) afforded 5.45 g of pure (2Z,5Z,8Z)-undecatrien-1-ol (46.9% yield); [$^1$H]NMR (90 MHz, $CDCl_3$): δ 5.80–5.14 (6H, m, vinyl protons), 4.20 (2H, d, $J$ = 6.0 Hz, $-C\underline{H}_2-OH$), 2.80 (4H, m, bisallylic methylenes), 2.03 (2H, m, $CH_3-C\underline{H}_2$), 1.37 (1H, bs, $-OH$), 0.91 (3H, t, $J$ = 7.5 Hz, $CH_3-$).

*(2S,3R,5Z,8Z)-2,3-Epoxy-5,8-undecadien-1-ol (Va).* To 175 ml of dry dichloromethane maintained at −23°C under argon was added titanium tetraisopropoxide (7.43 ml, 25 mmol), (+)-diisopropyl-L-tartrate (5.25 ml, 25 mmol), (2Z,5Z,8Z)-undecatrien-1-ol (3.98 g, 25 mmol) and, after 10 min, 8.9

ml of a 5.65 M solution of *t*-butylhydroperoxide in dichloromethane [prepared by the procedure of Sharpless and Verhoeven (1979)]. The reaction mixture was kept in a freezer at −20°C for 24 hr and then treated with 75 ml of 10% aqueous tartaric acid. The mixture was stirred vigorously until it reached 21°C and then transferred to a separatory funnel. The dichloromethane layer was separated and the aqueous phase extracted twice with 75 ml of dichloromethane. Product isolation yielded epoxyalcohol (Va) together with (+)-diisopropyl-L-tartrate. Removal of the tartrate ester was accomplished by stirring an ice-cold ether solution of the crude product with 60 ml of 1 N sodium hydroxide for exactly 15 min. Extractive work-up afforded crude Va which was purified by flash chromatography (25-cm × 4-cm-ID column, 20-ml fractions). Elution with hexane–ethyl acetate (2:1) gave 2.78 g (83.0% yield) of (2*S*,3*R*,5*Z*,8*Z*)-2,3-epoxy-5,8-undecadien-1-ol which was > 98% pure by GC analysis; [¹H]NMR (360 MHz, $CDCl_3$): δ 5.52 (1H, dtt, *J* = 10.7, 7.3, and 1.5 Hz, $-C_6\underline{H}=C_5H-$), 5.40 (2H, m, $-C_8\underline{H}=C_9\underline{H}$), 5.27 (1H, dtt, *J* = 10.7, 7.3, and 1.5 Hz, $-C_6H=C_5\underline{H}-$), 3.84 (1H, ddd, *J* = 12.1, 7.1, and 4.3 Hz, $-\underset{|}{C}H_B-OH$ with $\underline{H}_A$), 3.71 (1H, ddd, *J* = 12.1, 6.5, and 5.3 Hz, $-\underset{|}{C}H_A-OH$ with $\underline{H}_B$), 3.15 (1H, ddd, *J* = 6.5, 4.3, and 4.3, Hz $-C_3H-\overset{O}{\frown}C_2\underline{H}-$), 3.05 (1H, ddd, *J* = 6.5, 6.5, and 4.3 Hz, $-C_3\underline{H}-\overset{O}{\frown}C_2H-$), 2.78 (2H, dd, *J* = 7.1 and 7.1 Hz, $-CH=CH-C\underline{H}_2-CH=CH-$), 2.45 (1H,m, $-\underset{|}{C}_4H_B-$ with $\underline{H}_A$), 2.25 (1H, m, $-\underset{|}{C}_4H_A-$ with $\underline{H}_B$), 2.05 (2H, dq, *J* = 7.5 and 7.5 Hz, $CH_3C\underline{H}_2$), 1.72 (1H, dd, *J* = 7.1 and 5.3 Hz, $-O\underline{H}$), 0.96 (3H, t, *J* = 7.5 Hz, $C\underline{H}_3-$); $[\alpha]_D^{23}$ = −11.9° (C = 9.24, $CHCl_3$).

*(2*R*,3*S*,5*Z*,8*Z*)-2,3-Epoxy-5,8-undecadien-1-ol (Vb).* The procedure used for the preparation of this compound was identical to that described for the 2*S*,3*R* enantiomer (Va) except that (−)-diisopropyl-D-tartrate was employed in the epoxidation step. Flash chromatography afforded 4.75 g of the 2*R*,3*S* enantiomer (87.0% yield); $[\alpha]_D^{23}$ = 11.3° (C = 9.44, $CHCl_3$); the 360 MHz [¹H]NMR spectrum of the 2*R*,3*S* enantiomer was identical to that of the 2*S*,3*R* enantiomer.

*(2*S*,3*R*,5*Z*,8*Z*)-1-Tosyloxy-5,8-undecadiene (VIa) and (2*R*,3*S*,5*Z*,8*Z*)-1-Tosyloxy-5,8-undecadiene (VIb).* Tosylations of Va and Vb were carried out under identical conditions. To an ether solution (−10°C) of the epoxyalcohol and *p*-TsCl (1.05 equivalents) was added three equivalents of freshly powdered potassium hydroxide. After 45 min, extractive work-up yielded the crude product which was purified by flash chromatography (20 cm × 4.0 cm ID; hexane–ethyl acetate, 7:1) to afford 93.5% yields of each tosylate: (2*S*,3*R*,5*Z*,8*Z*)-1-tosyloxy-5,8-undecadiene (VIa); [¹H]NMR (90 MHz, $CDCl_3$): δ 7.83 (2H, d, *J* =

9.0 Hz, aromatic), 7.37 (2H, d, $J$ = 9.0 Hz, aromatic), 5.64–5.06 (4H, m, vinyl protons), 4.13 (2H, m, $-C\underline{H}_2-OTs$), 3.20–2.87 (2H, m, epoxide protons), 2.70 (2H, m, $-CH=CH-C\underline{H}_2-CH=CH-$), 2.40 (3H, s, $C\underline{H}_3-C_6H_4-$), 2.30–1.86 (4H, m, allylic protons), 0.93 (3H, t, $J$ = 7.5 Hz, $C\underline{H}_3-CH_2-$); $[\alpha]_D^{23}$ = −22.6° (C = 10.2, $CHCl_3$). (2*R*,3*S*,5*Z*,8*Z*)-1-tosyloxy-5,8-undecadiene (VIb); 90-MHz [$^1$H]NMR identical to spectrum of 2*S*,3*R* enantiomer; $[\alpha]_D^{23}$ = 23.5° (C = 10.4, $CHCl_3$).

*(3Z,6Z,9S,10R)-9,10-Epoxyheneicosadiene.* The procedure for preparation of the title compound [(3*Z*,6*Z*)-9*S*,10*R*-epoxy-21 : H], the saltmarsh caterpillar pheromone, is representative of the preparations of all the homologous epoxydienes and was carried out as follows: To a freshly prepared ether solution of lithium-di-*n*-decylcuprate (2.4 mmol), maintained under nitrogen at −25°C, was added 685 mg (2.04 mmol) of VIb in 5 ml of anhydrous ether. After 20 min, the reaction was quenched with 50 ml of saturated aqueous ammonium chloride and the crude product obtained by ether extraction (3 × 50 ml). Purification was achieved by HPLC on a Magnum ODS-3 column (Whatman). Gradient elution from methanol–water (75 : 25) to 100% methanol over 20 min at 4 ml/min afforded 401 mg (64% yield) of (3*Z*,6*Z*)-9*S*,10R-epoxyheneicosadiene which was greater than 98% pure by GC analysis; [$^1$H]NMR (90 MHz, $CDCl_3$): δ 5.64–5.10 (4H, m, vinyl protons), 3.0–2.7 (4H, m, epoxide and bis-allylic protons), 2.3 (2H, m, $-CH=CH-C\underline{H}_2-\overset{O}{CH-CH}-$), 2.03 (2H, m, $CH_3C\underline{H}_2$), 1.47 (2H, m, $-\overset{O}{CH-CH}-C\underline{H}_2-CH_2-$), 1.26 (18H, bs, $CH_3(C\underline{H}_2)_9-$), 0.93 (3H, t, $J$ = 7.5 Hz, $C\underline{H}_3CH_2CH=CH-$), 0.86 (3H, deformed t, $J$ = 7.5 Hz, $C\underline{H}_3(CH_2)_9-$); $[\alpha]_D^{23}$ = 3.4° (C = 8.67, $CHCl_3$). The 90-MHz [$^1$H]NMR spectrum reported above is virtually identical to the 100-MHz spectrum ($C_6D_6$) reported by Hill and Roelofs (1981) for the saltmarsh caterpillar pheromone.

Satisfactory spectral analyses were obtained on the higher ($C_{22}$) and lower ($C_{18}$–$C_{20}$) homologs of (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21 : H prepared under similar conditions with the appropriate cuprate reagent. The specific rotations for the homologs are as follows: (3*Z*,6*Z*)-9*S*,10*R*-epoxy-18 : H, $[\alpha]_D^{23}$ = 4.8° (C = 7.94, $CHCl_3$); (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19 : H, $[\alpha]_D^{23}$ = 3.5° (C = 8.57, $CHCl_3$); (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20 : H, $[\alpha]_D^{23}$ = 4.3° (C = 8.28, $CHCl_3$); (3*Z*,6*Z*)-9*S*,10*R*-epoxy-22 : H, $[\alpha]_D^{23}$ = 2.5° (C = 8.47, $CHCl_3$).

The homologous series of (3*Z*,6*Z*)-9*R*,10*S*-epoxydienes were prepared in identical manner to the 9*S*,10*R* enantiomers except that (2*S*,3*R*,5*Z*,8*Z*)-1-tosyloxy-2,3-epoxy-5,8-undecadiene was employed in the cuprate coupling reaction instead of the (2*R*,3*S*)-tosylate. The specific rotations of the products are as follows: (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18 : H, $[\alpha]_D^{23}$ = −4.2° (C = 5.74, $CHCl_3$); (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19 : H, $[\alpha]_D^{23}$ = −4.3° (C = 8.89, $CHCl_3$);

(3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H, $[\alpha]_D^{23}$ = −3.7° (C = 12.7, $CHCl_3$); (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21:H, $[\alpha]_D^{23}$ = −3.4° (C = 15.9, $CHCl_3$); (3*Z*,6*Z*)-9*R*,10*S*-epoxy-22:H, $[\alpha]_D^{23}$ = −1.7° (C = 8.26, $CHCl_3$).

*Field Trapping.* Experiments were performed with Pherocon 1CP® traps (Zoecon Corp., Palo Alto, California) baited with 500 μg of synthetic chemicals impregnated in rubber septa (A. H. Thomas, No. 8753-D22).

Twenty-one synthetic compounds or mixtures of compounds consisting of (3*Z*,6*Z*,9*Z*,11*E*)-19:H, the $C_{18}$–$C_{22}$ homologs of (3*Z*,6*Z*,9*Z*)-trienes, combined monoepoxides of (3*Z*,6*Z*,9*Z*)-trienes, (3*Z*,6*Z*)-9*S*,10*R*-epoxydienes, and (3*Z*,6*Z*)-9*R*,10*S*-epoxydienes were used in our experiments. In the field survey these compounds were set out individually and as binary mixtures which comprise the 118 different treatments shown in Table 2. Field survey treatments were duplicated and set out in complete randomized block designs at two locations 5 km apart. Traps were hung in a line on tree branches approximately 1.5 m above ground and at least 20 m apart and tended twice a week between May and September 1983. Captured specimens were identified at Biosystematics Research Institute, Ottawa, Canada. Similar procedures were followed for specific experiments except that treatments were 3X replicated.

Trap capture data were transformed $[(x + 0.5)^{1/2}]$, subjected to analysis of variance, and the means compared by Duncan's new multiple-range test.

*Electroantennography (EAG).* EAG measurements were performed as previously described (Chisholm et al., 1975). The compounds tested include all those used in the field survey, as described above, and the following groups; saturated and monounsaturated, $C_{10}$, $C_{12}$, $C_{14}$, $C_{16}$ acetates, alcohols, and aldehydes with *Z* and *E* unsaturation ranging from 5 to 11; saturated hydrocarbons $C_{11}$–$C_{24}$; (6*Z*,9*Z*)-diene hydrocarbons, $C_{18}$–$C_{22}$; (6*Z*,9*Z*,12*Z*)-triene hydrocarbons, $C_{18}$–$C_{22}$, and linolenyl acetate, alcohol, and aldehyde.

## RESULTS

Stereospecific synthesis of (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxyheneicosadiene was achieved in six steps from I with an overall yield of 12.5%. Although our synthetic route is similar to one reported by Mori and Ebata (1981), we obtained a much higher yield; 23.5% from III compared to less than 6% from a similar triyne intermediate in Mori and Ebata's synthesis. Improvements in yield were mainly due to our use of dicyclohexylborane for triyne reduction instead of P-2 nickel catalyst which is unsuitable for triynes (Huang et al., 1983). We also obtained significantly better yields for the asymmetric epoxidation and lithium dialkylcuprate coupling reactions. On the basis of the specific rotations of the key epoxyalcohol intermediates (Va and Vb), the enantiomeric purities of our products are higher than those prepared by Mori and Ebata. High-field [$^1$H]NMR

TABLE 2. COMPOUNDS AND BLENDS USED AS TRAP LURES IN 1983 FIELD SURVEY[a]

| Compound/blend | Compound/blend |
|---|---|
| (3Z,6Z,9Z)-18:H | CME-(3Z,6Z,9Z)-21:H |
| +(3Z,6Z,9Z)-19:H | +CME-(3Z,6Z,9Z)-22:H |
| +CME-(3Z,6Z,9Z)-18:H | CME-(3Z,6Z,9Z)-22:H |
| +(3Z,6Z)-9R,10S-epoxy-18:H | (3Z,6Z)-9R,10S-epoxy-18:H |
| +(3Z,6Z)-9S,10R-epoxy-18:H | +(3Z,6Z)-9R,10S-epoxy-19:H |
| (3Z,6Z,9Z)-19:H | (3Z,6Z)-9R,10S-epoxy-19:H |
| +(3Z,6Z,9Z)-20:H | +(3Z,6Z)-9R,10S-epoxy-20:H |
| +CME-(3Z,6Z,9Z)-19:H | (3Z,6Z)-9R,10S-epoxy-20:H |
| +(3Z,6Z)-9R,10S-epoxy-19:H | +(3Z,6Z)-9R,10S-epoxy-21:H |
| +(3Z,6Z)-9S,10R-epoxy-19:H | (3Z,6Z)-9R,10S-epoxy-21:H |
| (3Z,6Z,9Z)-20:H | +(3Z,6Z)-9R,10S-epoxy-22:H |
| +(3Z,6Z,9Z)-21:H | (3Z,6Z)-9R,10S-epoxy-22:H |
| +CME-(3Z,6Z,9Z)-20:H | (3Z,6Z)-9S,10R-epoxy-18:H |
| +(3Z,6Z)-9R,10S-epoxy-20:H | +(3Z,6Z)-9S,10R-epoxy-19:H |
| +(3Z,6Z)-9S,10R-epoxy-20:H | (3Z,6Z)-9S,10R-epoxy-19:H |
| (3Z,6Z,9Z)-21:H | +(3Z,6Z)-9S,10R-epoxy-20:H |
| +(3Z,6Z,9Z)-22:H | (3Z,6Z)-9S,10R-epoxy-20:H |
| +CME-(3Z,6Z,9Z)-21:H | +(3Z,6Z)-9S,10R-epoxy-21:H |
| +(3Z,6Z)-9R,10S-epoxy-21:H | (3Z,6Z)-9S,10R-epoxy-21:H |
| +(3Z,6Z)-9S,10R-epoxy-21:H | +(3Z,6Z)-9S,10R-epoxy-22:H |
| (3Z,6Z,9Z)-22:H | (3Z,6Z)-9S,10R-epoxy-22:H |
| +CME-(3Z,6Z,9Z)-22:H | (3Z,6Z,9Z,11E)-19:H |
| +(3Z,6Z)-9R,10S-epoxy-22:H | +(3Z,6Z,9Z)-19:H |
| +(3Z,6Z)-9S,10R-epoxy-22:H | (3Z,6Z)-9S,10R-epoxy-20:H |
| CME-(3Z,6Z,9Z)-18:H | +(3Z,6Z)-9R,10S-epoxy-20:H |
| +CME-(3Z,6Z,9Z)-19:H | |
| CME-(3Z,6Z,9Z)-19:H | |
| +CME-(3Z,6Z,9Z)-20:H | |
| CME-(3Z,6Z,9Z)-20:H | |
| +CME-(3Z,6Z,9Z)-21:H | |

[a]Compounds tested as single components are underlined. Binary blends consisted of the underlined compound and each of the compounds listed below at ratios of 1:4, 1:1 and 4:1. All lures contained a total of 500 μg.

analysis of the (+)-$\alpha$-methoxy-$\alpha$-(trifluoromethyl)phenyl acetate derivatives of Va and Vb revealed enantiomeric excesses of 93% and 92%, respectively.

During 1982, a preliminary field screening of unsaturated hydrocarbons, combined monoepoxides of (3*Z*,6*Z*,9*Z*)-trienes, and linolenyl derivatives was performed. Field captures and strong EAG responses by male geometrids and noctuids, primarily to (3*Z*,6*Z*,9*Z*)-trienes and the combined monoepoxydiene derivatives, suggested the importance of these compounds as sex attractants (Underhill et al., unpublished). Consequently, we focused our 1983 field survey on these compounds together with two homologous series of enantiomerically enriched (3*Z*,6*Z*)-*cis*-9,10-epoxydienes ($C_{18}$–$C_{22}$). A component of the fall cankerworm moth pheromone, (3*Z*,6*Z*,9*Z*,11*E*)-19 : H, was added to the survey following its identification and synthesis (Wong et al., 1984a,b). The other unsaturated hydrocarbons and linolenyl derivatives described in Methods and Materials were only used for EAG assay.

Between May and September 1983, males belonging to 17 species of Geometridae were captured in our traps. All of these species belonged to the subfamilies Larentiinae or Ennominae which make up 87% of the Geometridae of North America north of Mexico (Hodges et al., 1983). Males of nine noctuid species from the subfamilies Catocalinae, Herminiinae, Hypeninae, and Rivulinae were also captured. Multiple captures of conspecific males to specific lures were recorded for several species from both families. Prior to discussions of individual species, it is important to note, with the methodology employed here, that comparisons of lure attractancy to virgin females were not possible. Since we had no information on the population size of the various species, the potency of our attractants could only be judged on a relative basis. Low numbers of insects caught may be an indication of poor attractancy or a low population of the species in our region.

Only one species, the geometrid *Epirrhoe sperryi*, responded to a (3*Z*,6*Z*,9*Z*)-triene as a single-component lure. In 1982, Pherocon 1CP traps baited with 500, 200, and 50 $\mu$g of (3*Z*,6*Z*,9*Z*)-19 : H caught 35, 22 and 4 *E. sperryi* males, respectively, in a 3X replicated experiment (June 21–July 12, 1982). These results were confirmed and expanded by data obtained during the 1983 field survey (Table 3). The only single-component lure which effectively produced multiple captures of *E. sperryi* was (3*Z*,6*Z*,9*Z*)-19 : H. Captures of *E. sperryi* males by (3*Z*,6*Z*,9*Z*)-19 : H were unaffected by (3*Z*,6*Z*,9*Z*)-18 : H at the 4 : 1 ratio, but greatly reduced or completely blocked by various doses of (3*Z*,6*Z*,9*Z*)-20 : H. The apparent synergistic effect of (3*Z*,6*Z*,9*Z*,11*E*)-19 : H was not confirmed by a second experiment. The reduction in total males captured between the 4 : 1 and 1 : 1 ratios of (3*Z*,6*Z*,9*Z*)-19 : H plus (3*Z*,6*Z*,9*Z*)-18 : H, CME-(3*Z*,6*Z*,9*Z*)-19 : H, or (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19 : H cannot be clearly attributed to either dose of (3*Z*,6*Z*,9*Z*)-19 : H or effect of the second component since a comparison of dose of (3*Z*,6*Z*,9*Z*)-19 : H versus captures of *E. sperryi*

TABLE 3. CAPTURE OF *Epirrhoe sperryi* MALES IN SURVEY TRAPS

| Lure composition (μg) | Total males captured[a] |
|---|---|
| (3*Z*,6*Z*,9*Z*)-19:H (500) | 46 |
| (400) + (3*Z*,6*Z*,9*Z*)-18:H (100) | 42 |
| (250) + (3*Z*,6*Z*,9*Z*)-18:H (250) | 3 |
| (400) + (3*Z*,6*Z*,9*Z*)-20:H (100) | 4 |
| (400) + (3*Z*,6*Z*,9*Z*,11*E*)-19:H (100) | 73 |
| (250) + (3*Z*,6*Z*,9*Z*,11*E*)-19:H (250) | 23 |
| (400) + CME-(3*Z*,6*Z*,9*Z*)-19:H (100) | 47 |
| (250) + CME-(3*Z*,6*Z*,9*Z*)-19:H (250) | 9 |
| (100) + CME-(3*Z*,6*Z*,9*Z*)-19:H (400) | 7 |
| (400) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (100) | 52 |
| (250) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (250) | 16 |
| (100) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (400) | 13 |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (500) | 1 |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (100) | 7 |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (250) | 13 |
| (100) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (400) | 2 |

[a]Total males captured in 2X replicated survey traps between May 30 and June 27, 1983.

was not carried out. This comment applies in general to the other species captured and must be kept in mind during interpretation of results. Note here that in Table 3 and other tables, absence of an experimental treatment used in the survey indicates no captures of the species described in the Table heading. While additions of CME-(3*Z*,6*Z*,9*Z*)-19:H or (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H to (3*Z*,6*Z*,9*Z*)-19:H failed to produce any obvious effects, (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H appeared to reduce captures of *E. sperryi*. However, this effect was not observed during a 3X replicated experiment designed to test the effect of various doses of epoxydienes (data not shown).

Thus the effect of epoxides, if any, on the attractancy of (3*Z*,6*Z*,9*Z*)-19:H to *E. sperryi* remains unclear. An EAG assay was performed on *E. sperryi* in 1982 with all of the compounds described in Methods and Materials except for (3*Z*,6*Z*,9*Z*,11*E*)-19:H and the enantiomers of (3*Z*,6*Z*)-*cis*-9,10-epoxydienes. Responses were strongest for (3*Z*,6*Z*,9*Z*)-19:H and CME-(3*Z*,6*Z*,9*Z*)-19:H and adjacent higher and lower homolog (Figure 1). Although not shown in Figure 1, EAG responses by *E. sperryi* were near background for all of the other groups of compounds used in electroantennography, including the saturated and monounsaturated acetates, alcohols and aldehydes, saturated hydrocarbons, (6*Z*,9*Z*)-diene hydrocarbons, (6*Z*,9*Z*,12*Z*)-triene hydrocarbons, and linolenyl derivatives. This statement also applies to the other geometrid and noctuid moths captured in the survey (Figures 2–5). EAG responses were elicited principally by the (3*Z*,6*Z*,9*Z*)-triene hydrocarbons, their combined monoepoxydiene analogs, and the (9*S*,10*R*)- and (9*R*,10*S*)-monoepoxydienes. In general, those

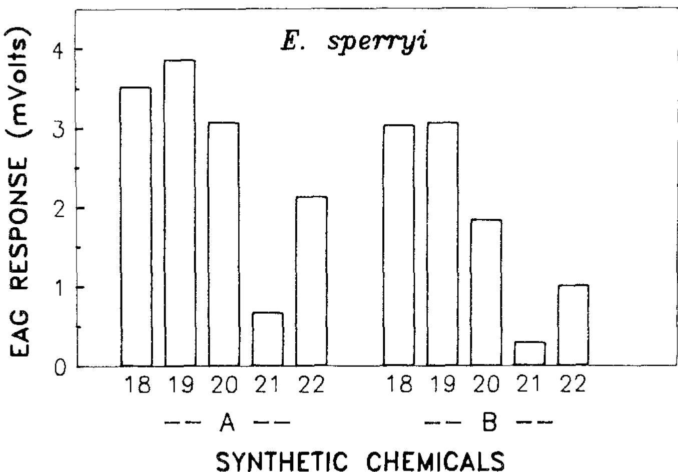


FIG. 1. EAG responses of *E. sperryi* to synthetic chemicals. Letters (A and B) refer to (3Z,6Z,9Z)-*X*: H and CME-(3Z,6Z,9Z)-*X*: H, respectively. Numbers (18–22) refer to the length of the carbon chain (*X*).

compounds which attracted moths in the field also elicited the strongest EAG signals. Recently, (3*Z*,6*Z*,9*Z*)-19:H has been identified as a pheromone component for the giant looper, *Boarmia (ascotis) selenaria* (Becker et al., 1983), the spring cankerworm, *Paleacrita vernata* (Underhill et al., unpublished), and the fall cankerworm moth, *Alsophila pometaria* (Wong et al., 1984a).

Two geometrid species, *Mesoleuca ruficillata* and *Triphosa haesitata affirmata* (Guenée), both of the Hydriomenini tribe, were captured by lures consisting predominantly of (3*Z*,6*Z*,9*Z*)-trienes. Single captures of *M. ruficillata* occurred with five binary lures in which the principal components were (3*Z*,6*Z*,9*Z*)-19:H, (3*Z*,6*Z*,9*Z*)-20:H, or (3*Z*,6*Z*,9*Z*)-21:H. The minor components of these lures did not share any obvious common features. The best lure for *M. ruficillata*, (3*Z*,6*Z*,9*Z*)-21:H (400 μg) + (3*Z*,6*Z*,9*Z*)-22:H (100 μg), captured five males, and also captured one of the two specimens of *T. haesitata affirmata* taken during 1983. The other specimen was caught in a survey trap baited with (3*Z*,6*Z*,9*Z*)-21:H (450 μg) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (50 μg). Although these captures alone are not conclusive, data from 1982 suggest (3*Z*,6*Z*,9*Z*)-trienes may be components of an attractant. Survey traps baited with (3*Z*,6*Z*,9*Z*)-20:H (200 μg) + linolenyl aldehyde (10 μg), (3*Z*,6*Z*,9*Z*)-20:H (200 μg) + linolenyl acetate (10 μg), and (3*Z*,6*Z*,9*Z*)-21:H (200 μg) + linolenyl acetate (10 μg) captured two, seven, and five males of *T. haesitata affirmata*, respectively, between May 18 and June 9, 1982. These results suggest that a combination of a (3*Z*,6*Z*,9*Z*)-triene ($C_{20}$ and/or $C_{21}$) and linolenyl acetate may be required for attraction of *T. haesitata affirmata*.

TABLE 4. CAPTURE OF *Metanema inatomaria* AND *Prochoerodes transversata* MALES IN SURVEY TRAPS

| Lure composition (μg) | Total males captured | |
|---|---|---|
| | *M. inatomaria*[a] | *P. transversata*[b] |
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-18:H (400) + (3*Z*,6*Z*,9*Z*)-18:H (100) | 0 | 1 |
| (250) + (3*Z*,6*Z*,9*Z*)-18:H (250) | 0 | 1 |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H (500) | 7ef | 1 |
| (400) + (3*Z*,6*Z*,9*Z*)-18:H (100) | 31de | 0 |
| (250) + (3*Z*,6*Z*,9*Z*)-18:H (250) | 6f | 0 |
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (400) + (3*Z*,6*Z*,9*Z*)-19:H (100) | 0 | 56 |
| (250) + (3*Z*,6*Z*,9*Z*)-19:H (250) | 0 | 47 |
| (100) + (3*Z*,6*Z*,9*Z*)-19:H (400) | 0 | 7 |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (500) | 80bc | 0 |
| (400) + (3*Z*,6*Z*,9*Z*)-19:H (100) | 2f | 1 |
| (250) + (3*Z*,6*Z*,9*Z*)-19:H (250) | 0 | 4 |
| (400) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H (100) | 85bc | 0 |
| (250) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H (250) | 38cd | 0 |
| (100) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H (400) | 11def | 0 |
| (400) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (100) | 116ab | 0 |
| (250) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (250) | 44c | 0 |
| (100) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (400) | 6ef | 0 |
| (400) + (3*Z*,6*Z*,9*Z*,11*E*)-19:H (100) | 169a | 0 |
| (250) + (3*Z*,6*Z*,9*Z*,11*E*)-19:H (250) | 101ab | 0 |
| (100) + (3*Z*,6*Z*,9*Z*,11*E*)-19:H (400) | 48c | 0 |

[a] Total males captured in 2X replicated Pherocon 1CP traps between June 7 and July 25, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).
[b] Total males captured in 2X replicated Pherocon 1CP traps between June 17 and September 7, 1983.

Several species of geometrids were specifically attracted to survey traps baited with an epoxide as the major component. Captures of *Metanema inatomaria* and *Prochoerodes transversata* clearly illustrate this attraction (Table 4). *M. inatomaria* males were caught by (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H and the less potent (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H as single-component lures. The specificity of this response is shown by the absence of captures to the opposite (9*S*,10*R*)-epoxydiene isomers. (3*Z*,6*Z*,9*Z*)-Trienes appeared to have opposite effects on the two epoxydiene attractants. Although not statistically significant, (3*Z*,6*Z*,9*Z*)-18:H appeared to be synergistic with (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H, while (3*Z*,6*Z*,9*Z*)-19:H blocked the attraction of *M. inatomaria* to (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H. Combination of the two attractive epoxydienes did not cause a synergistic response. Two compounds appeared to increase the attractancy of (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H. Captures of *M. inatomaria* males were increased slightly by (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H, while addition of (3*Z*,6*Z*,9*Z*,11*E*)-19:H produced a significant twofold increase. The synergistic effect of (3*Z*,6*Z*,9*Z*,11*E*)-19:H was confirmed by a 3X replicated experiment conducted between June 21 and July 7, 1983. Traps baited with (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H and (3*Z*,6*Z*,9*Z*,11*E*)-19:H, (400:100 μg) and (475:25 μg), captured 83 and 60 males of *M. inatomaria*, respectively, compared to 16 males caught by 500 μg of the epoxydiene alone.

Another experiment revealed that the attractancy of (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H was blocked by its enantiomer or the combined monoepoxides of (3*Z*,6*Z*,9*Z*)-19:H (Table 5). No males of *M. inatomaria* were captured by a 1:1 (racemic) mixture of (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H and its (9*S*,10*R*)-enantiomer. Inhibition by *CME*-(3*Z*,6*Z*,9*Z*)-19:H is complete at the 25-μg dose, which indicates that one or both of the other isomers, (3*Z*,9*Z*)-*cis*-6,7-epoxy-

TABLE 5. EFFECT OF DOSE OF (3*Z*,6*Z*)-9*R*,10*S*-EPOXY-19:H, (3*Z*,6*Z*)-9*S*,10*R*-EPOXY-19:H, AND CME-(3*Z*,6*Z*,9*Z*)-19:H ON CAPTURES OF *Metanema inatomaria*

| Lure composition (μg) | Total males captured[a] |
|---|---|
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (500) | 35a |
| (250) | 25ab |
| (50) | 7bc |
| (475) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (25) | 66a |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (100) | 40ab |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (250) | 0c |
| (475) + CME-(3*Z*,6*Z*,9*Z*)-19:H (25) | 0c |

[a]Total males captured in 3X replicated Pherocon 1CP traps between June 17 and July 7, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).

19:H or (6*Z*,9*Z*)-*cis*-3,4-epoxy-19:H, is responsible, since the quantity of (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H present (4.2 μg) is insufficient for complete inhibition. The antennal responses of *M. inatomaria* to synthetic compounds (Figure 2) were consistent with field capture data and were strong for compounds which are attractants, synergists, and inhibitors. It is interesting to note that

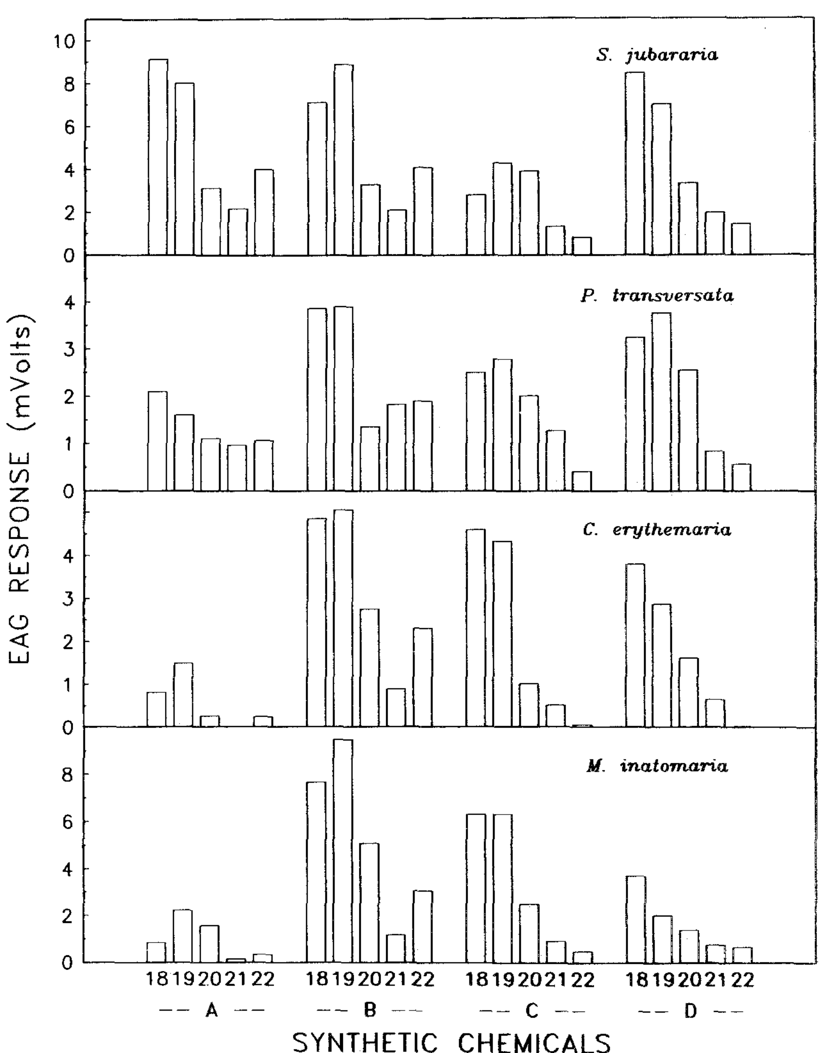


FIG. 2. EAG responses of *M. inatomaria, C. erythemaria, P. transversata*, and *S. jubararia* to synthetic chemicals. Letters (A–D) refer to (3Z,6Z,9Z)-*X*:H, CME-(3Z,6Z,9Z)-*X*:H, (3Z,6Z)-9*R*,10*S*-epoxy-*X*:H, and (3Z,6Z)-9*S*,10*R*-epoxy-*X*:H, respectively. Numbers (18–22) refer to the length of the carbon chain (*X*).

principal attractants, (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H and -18:H, elicited stronger antennal responses than the unattractive 9*S*,10*R* enantiomers. Hill et al. (1982) have reported that *Hyphantrea cunea* produces larger antennal responses to (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H than its 9*R*,10*S* enantiomer.

Another geometrid moth, *Cabera erythemaria*, gave an EAG profile (Figure 2) very similar to that of *M. inatomaria*. This moth was not attracted by any of the lures used in the field survey and was captured in experiments designed to determine the effect of lure composition on captures of *M. inatomaria*. Lures composed of (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H and (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H, 475:25 μg and 400:100 μg, captured *M. inatomaria* males and no *C. erythemaria* males (Table 5) while a 1:1 mixture of these compounds captured 21 males of *C. erythemaria* and no *M. inatomaria*. This phenomenon of attraction of one species and inhibition of a second species by a specific ratio of enantiomers is similar to the situation reported for the responses of *Lymantria dispar* and *L. monacha* to (+)- and (−)-disparlure mixtures (Hansen et al., 1983). Further experiments disclosed that (3*Z*,6*Z*,9*Z*)-19:H was required for improved captures of *C. erythemaria*. The addition of 10 μg and 50 μg of (3*Z*,6*Z*,9*Z*)-19:H to a racemic mixture of the 9*S*,10*R* and 9*R*,10*S* enantiomers (500 μg) led to captures of 71 and 34 males, respectively. These captures were significantly greater ($P = 0.05$) than the capture of three males by a treatment consisting of 500 μg of the racemate alone (3X replicated, July 11–18, 1983).

The large maple spanworm, (*Prochoerodes transversata*), is commonly found in oak and maple and is distributed throughout the temperate regions of North America. In our region, two flights occur during June–July and August–September. While single captures of *P. transversata* were recorded for several survey lures, most males were caught by binary mixtures of (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H and (3*Z*,6*Z*,9*Z*)-19:H (Table 4). The absence of captures for either compound alone indicated an absolute requirement for a combination of both compounds. This was confirmed by a 3X replicated experiment (August 18–September 4, 1983) which resulted in captures of 41 and 54 males in traps baited with (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H and (3*Z*,6*Z*,9*Z*)-19:H at doses of 250:250 μg and 400:100 μg compared to no captures for 500:10 μg and 500:1 μg mixtures.

Single captures of *P. transversata* by mixtures of (3*Z*,6*Z*)-9*S*,10*R*-epoxy-18:H + (3*Z*,6*Z*,9*Z*)-18:H may be indicative of weak activity by these compounds (Table 4). Weak attraction by lower homologs of principal attractants was also observed for *M. inatomaria* and could suggest a certain degree of flexibility in the specificity of receptor sites for the principal attractants that allows lower homologs to fit into these sites. Captures of *P. transversata* by lures containing (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H may be due to weak activity by the 9*R*,10*S* enantiomer or to the 9*S*,10*R* isomer present in the sample. However, the small quantity of the 9*S*,10*R* isomer present (3.5%) makes the latter expla-

nation unlikely. The EAG profile for *P. transversata* (Figure 2) contained strong responses to compounds identified as attractants or inhibitors by the field experiments. Note that the response is higher for the more attractive (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H compared to the 9*R*,10*S* isomer.

*Synaxis jubararia*, which belongs to the same tribe (Ourapterygini) as *P. transversata*, gave strong antennal responses to the compounds which also stimulated *P. transversata* (Figure 2). In survey traps, four, one, two, and two males of *S. jubararia* were captured in traps baited with (3*Z*,6*Z*,9*Z*)-19:H (400 μg) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (100 μg), (3*Z*,6*Z*,9*Z*)-19:H (250 μg) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (250 μg), (3*Z*,6*Z*,9*Z*)-19:H (400 μg) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (100 μg), and (3*Z*,6*Z*,9*Z*)-19:H (400 μg) + CME-(3*Z*,6*Z*,9*Z*)-19:H (100 μg), respectively. These captures suggest that (3*Z*,6*Z*,9*Z*)-19:H, as a major component, together with one enantiomer or a specific combination of both enantiomers of (3*Z*,6*Z*)-*cis*-9,10-epoxy-19:H may be required for attraction of *S. jubararia*.

Another geometrid moth captured by lures consisting solely or principally of an epoxide was *Dysstroma brunneata ethela*. Lures consisting only of (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H or (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H captured *D. brunneata ethela* males (Table 6). Captures of *D. brunneata ethela* by the $C_{20}$ epoxide were completely blocked by addition by of (3*Z*,6*Z*,9*Z*)-20:H but were not obviously affected by the other compounds tested in the survey, The EAG profile of *D. brunneata ethela* (Figure 3) is very consistent with the field trapping data and shows stronger responses for the attractive (9*S*,10*R*)-epoxydiene isomers than the inactive 9*R*,10*S* isomers.

Twelve specimens of a second *Dysstroma sp.*, tentatively identified as *citrata* (the dark marbled carpet moth), were captured in 10 traps baited with binary lures. All of the lures contained (3*Z*,6*Z*,9*Z*)-20:H or (3*Z*,6*Z*,9*Z*)-21:H plus one of the following: CME-(3*Z*,6*Z*,9*Z*)-20:H, CME-(3*Z*,6*Z*,9*Z*)-21:H, (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H, (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H, (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H, or (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21:H. Although a principal attractant was not discerned from the field-capture data, some combination of a triene hydrocarbon ($C_{20}$ and/or $C_{21}$) and an epoxydiene ($C_{20}$ and/or $C_{21}$) appears to be required.

Several noctuid moths were also specifically attracted by lures consisting primarily of an epoxide. Lures which contained (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H, alone or in a number of combinations, consistently attracted *Spargaloma sexpunctata* (subfamily Hypeninae). Table 7 summarizes the captures of this species in the survey (test 1) and one other field test. The greatest number of males captured was in traps baited with (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H alone. The attraction, which appeared to be stereospecific, was not enhanced by additions of other components and may have been inhibited by some. Although mixtures of the $C_{20}$ and $C_{21}$ combined monoexpoxydiene hydrocarbons captured a few

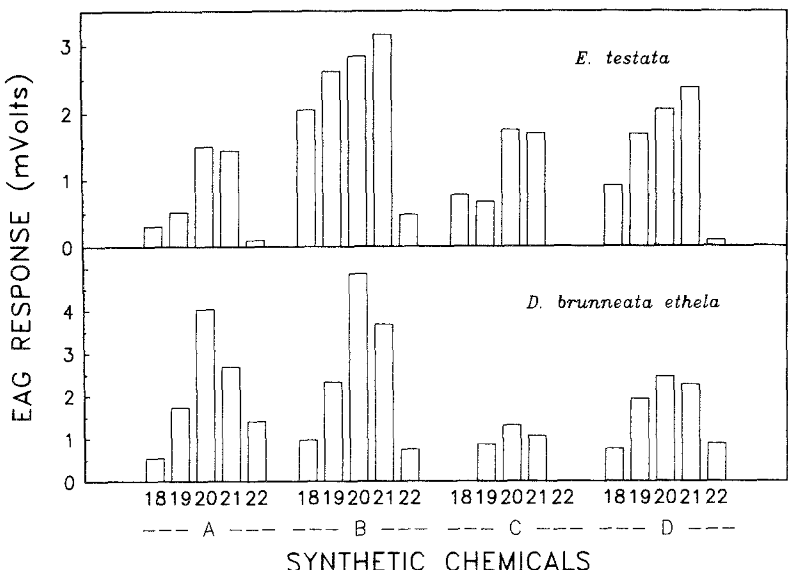


FIG. 3. EAG responses of *D. brunneata ethela* and *E. testata*. Letters (A–D) refer to (3Z,6Z,9Z)-*X*: H, CME-(3Z,6Z,9Z)-*X*: H, (3Z,6Z)-9*R*,10*S*-epoxy-*X*: H, and (3Z,6Z)-9*S*,10*R*-epoxy-*X*: H, respectively. Numbers (18–22) refer to the length of the carbon chain (*X*).

TABLE 6. CAPTURE OF *Dysstroma brunneata ethela* IN SURVEY TRAPS

| Lure composition (μg) | Total males captured[a] |
|---|---|
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H (500) | 41a |
| (250) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (250) | 21abc |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (100) | 14abc |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (250) | 6bc |
| (100) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (400) | 2c |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H (100) | 30ab |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H (250) | 30ab |
| (100) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H (400) | 21abc |
| (400) + (3*Z*,6*Z*,9*Z*)-20:H (100) | 0c |
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H (500) | 4bc |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-22:H (100) | 14abc |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-22:H (250) | 9abc |
| (100) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-22:H (400) | 4bc |

[a]Total males captured in 2× replicated Pherocon 1CP traps from July 11 to August 4, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).

TABLE 7. CAPTURE OF *Spargaloma sexpunctata* IN 1983 FIELD TESTS

| Lure composition (μg) | Males caught[a] | |
|---|---|---|
| | Test 1 | Test 2[b] |
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H (500) | 47 | 21a |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (100) | 20 | |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (250) | 7 | |
| (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H (100) | 17 | |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-21:H (250) | 4 | |
| (250) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (250) | 19 | |
| (450) + CME-(3*Z*,6*Z*,9*Z*)-20:H (50) | | 0b |
| (400) + CME-(3*Z*,6*Z*,9*Z*)-20:H (100) | 0 | 3b |
| (250) + (3*Z*,6*Z*,9*Z*)-20:H (250) | 4 | |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (500) | 3 | |
| (400) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21:H (100) | 5 | |
| CME-(3*Z*,6*Z*,9*Z*)-20:H (250) + CME-(3*Z*,6*Z*,9*Z*)21:H (250) | 10 | |

[a]Total number of target males captured. Test 1: field survey data from 2X replicated Pherocon 1CP traps. Moths were caught between June 14, and July 21, 1983. Test 2: 3X replicated Pherocon 1CP, July 10–18, 1983.
[b]Values followed by common letters do not differ at the 5% level.

specimens, CME-(3*Z*,6*Z*,9*Z*)-20:H alone failed to capture any and, in a 3X replicated test (test 2), its addition to (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H significantly decreased the number of males captured, suggesting one or more of the other isomers present in the mixture to be inhibitory. The capture or lack of capture of moths by several lure combinations are at present unaccountable and further field testing is required. This was particularly noticeable with binary mixtures of (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H plus (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H which, at 1:4 and 4:1, failed to capture any *S. sexpunctata* but at 1:1 (the racemate) was attractive. In response to the test series of synthetic compounds, major antennal stimulation was elicited by CME-(3*Z*,6*Z*,9*Z*)-20:H, (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H, and (3*Z*,6*Z*,9*Z*)-20:H; by comparison the response to the 9*R*,10*S* isomer was low (Figure 4).

Two other species within the Hypeninae subfamily were also captured and both appeared to respond to a specific epoxide. A total of 18 specimens of *Lomanaltes eductalis* were captured and all, except one, were attracted to (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21:H-based lures. No males were caught in CME-baited traps, which suggests that one or more of the isomeric monoepoxydienes in the mixtures was inhibitory. A small number (six) of a *Bomolocha* sp., [probably *palparia* (Walker)] were also captured. These were all taken by traps baited with the next lower homolog (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H.

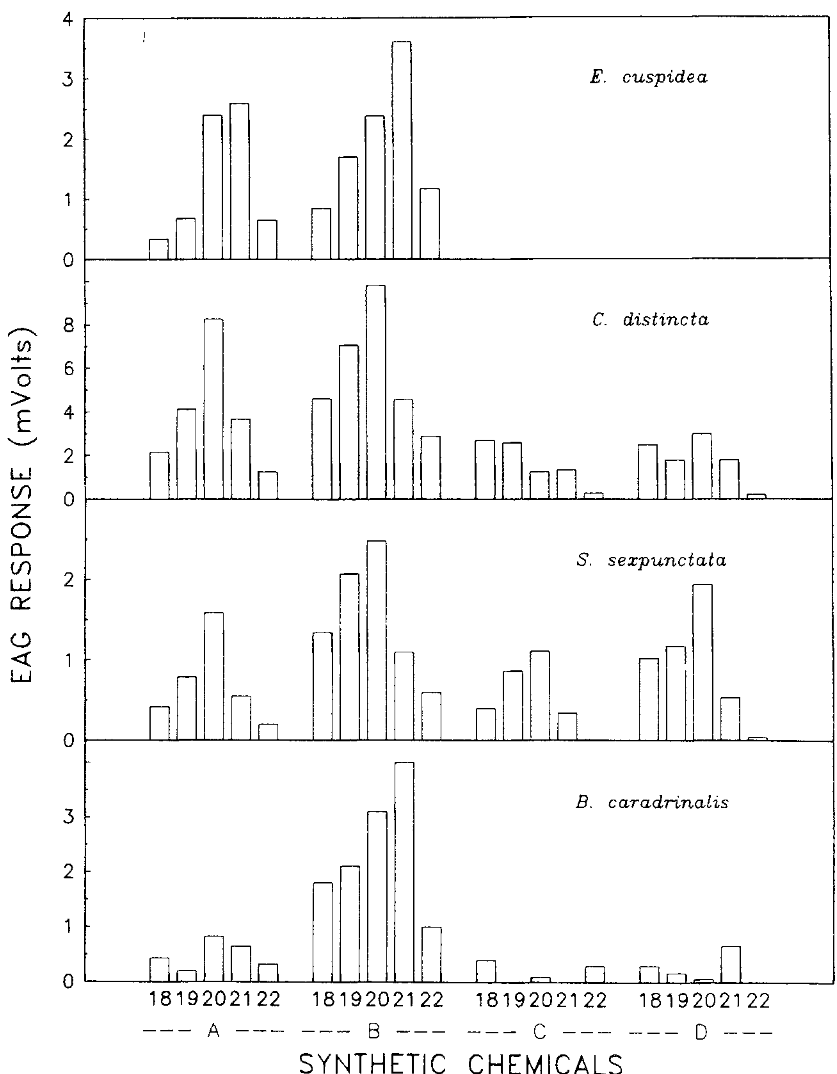


FIG. 4. EAG responses of *E. cuspidea, C. distincta, S. sexpunctata*, and *B. caradrinalis*. Letters (A–D) refer to (3*Z*,6*Z*,9*Z*)-*X*: H, CME-(3*Z*,6*Z*,9*Z*)-*X*: H, (3*Z*,6*Z*)-9*R*,10*S*-epoxy-*X*: H, and (3*Z*,6*Z*)-9*S*,10*R*-epoxy-*X*: H, respectively. Numbers (18–22) refer to the length of the carbon chain (*X*).

Several survey traps captured the noctuid *Caenurgina distincta*, subfamily Catocalinae (Table 8). Traps containing (3*Z*,6*Z*,9*Z*)-20:H alone took a few specimens. Blends of this hydrocarbon with its next lower or higher homologs produced no synergistic effects and the captures, which were all less than seven, were deleted from Table 8. However, binary lures containing (3*Z*,6*Z*,9*Z*)-20:H

TABLE 8. CAPTURE OF *Caenurgina distincta* MALES IN 1983 SURVEY TRAPS

| Lure composition ($\mu$g) | Males caught[a] |
|---|---|
| (3*Z*,6*Z*,9*Z*)-20:H (500) | 7 |
| (3*Z*,6*Z*,9*Z*)-20:H (400) + CME-(3*Z*,6*Z*,9*Z*)-20:H (100) | 33 |
| (250) + CME-(3*Z*,6*Z*,9*Z*)-20:H (250) | 35 |
| (100) + CME-(3*Z*,6*Z*,9*Z*)-20:H (400) | 14 |
| CME-(3*Z*,6*Z*,9*Z*)-20:H (500) | 3 |
| (3*Z*,6*Z*,9*Z*)-20:H (400) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (100) | 19 |
| (250) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (250) | 56 |
| (100) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (400) | 71 |
| (3*Z*,6*Z*,9*Z*)-20:H (400) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H (100) | 91 |
| (250) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H (250) | 113 |
| (100) + (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H (400) | 73 |
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H (500) | 2 |

[a]Total number of target males captured in 2X replicated Pherocon 1CP traps. Captures occurred between May 25, and July 18, 1983.

plus any one of CME-(3*Z*,6*Z*,9*Z*)-20:H, (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H, and (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H resulted in large increases in captures. Traps which contained these three additives as single components captured only a few males or none. The EAG response profile obtained with *C. distincta* males showed only two major peaks of activity in response to (3*Z*,6*Z*,9*Z*)-20:H and CME-(3*Z*,6*Z*,9*Z*)-20:H (Figure 4). Considering the field capture data, it is somewhat surprising that major antennal responses were not elicited by (3*Z*,6*Z*)-9*S*,10*R*-epoxy-20:H and (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H as well.

Two other species from the large Catocalinae subfamily were also captured in the field survey traps, *Euclidia cuspidea* and *Zale duplicata*. The EAG response profile of *E. cuspidea* (Figure 4) shows similarly strong responses were elicited by both the $C_{20}$ and $C_{21}$ (3*Z*,6*Z*,9*Z*)-triene hydrocarbons and their combined monoepoxydiene analogs. The (9*R*,10*S*)- and (9*S*,10*R*)-monoepoxydiene enantiomers were not available at the time these analyses were done. In our survey, *E. cuspidea* were only captured in traps baited with mixtures of the $C_{21}$ triene hydrocarbon and its epoxides. Of 45 specimens taken, 25 were attracted to mixtures of (3*Z*,6*Z*,9*Z*)-21:H + CME-(3*Z*,6*Z*,9*Z*)-21:H and the remainder to mixtures of the hydrocarbon and its (9*R*,10*S*)- and (9*S*,10*R*)-monoepoxydiene enantiomers. These results indicate that a combination of (3*Z*,6*Z*,9*Z*)-21:H and an epoxide are required for attraction of *E. cuspidea*. A total of 13 specimens of *Zale duplicata* were captured in the survey, and all but one were

taken by traps in which (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21 : H was the only or major component present.

Several noctuid species from the subfamilies Herminiinae and Rivulinae were captured in traps baited principally with combined monoepoxydiene mixtures. Three species from the Herminiinae were captured in the survey traps, namely *Bleptina caradrinalis*, *Idia americalis*, and *I. aemula*. *B. caradrinalis*, a noctuid widely distributed from central Canada to the Gulf of Mexico and westward to the Rocky Mountains, was regularly taken in traps baited with CME-(3*Z*,6*Z*,9*Z*)-21 : H (Table 9). The largest number of specimens was captured in traps baited with CME-(3*Z*,6*Z*,9*Z*)-21 : H alone. Only a few males were caught in traps baited with CME-(3*Z*,6*Z*,9*Z*)-20 : H alone and none by the $C_{22}$ homolog. The data indicate that none of the binary mixtures were synergistic in the three ratios tested. It is not known which of the *cis*-3,4-,*cis*-6,7- or *cis*-9,10-epoxides present in the CME mixture stimulated *B. caradrinalis* male attraction; however, no specimens were taken in traps containing either the $C_{21}$ 9*R*,10*S* or 9*S*,10*R* isomers as single components and neither elicited appreciably strong EAG responses (Figure 4).

EAG responses to the synthetic compounds listed in Methods and Materials were measured using *B. caradrinalis* males captured in the survey traps (Figure 4). Major responses were only elicited by the field-active compounds, CME-(3*Z*,6*Z*,9*Z*)-20 : H and CME-(3*Z*,6*Z*,9*Z*)-21 : H, the others giving lower or near background responses. Antennal responses to (*Z*)- and (*E*)-monounsat-

TABLE 9. CAPTURE OF *Bleptina caradrinalis* IN 1983 SURVEY TRAPS

| Lure composition (μg) | Males caught[a] |
|---|---|
| CME-(3*Z*,6*Z*,9*Z*)-21 : H (500)[b] | 46 |
| CME-(3*Z*,6*Z*,9*Z*)-21 : H (400) + (3*Z*,6*Z*,9*Z*)-21 : H (100) | 12 |
| (250) + (3*Z*,6*Z*,9*Z*)-21 : H (250) | 4 |
| (100) + (3*Z*,6*Z*,9*Z*)-21 : H (400) | 1 |
| CME-(3*Z*,6*Z*,9*Z*)-21 : H (400) + CME-(3*Z*,6*Z*,9*Z*)-20 : H (100) | 14 |
| (250) + CME-(3*Z*,6*Z*,9*Z*)-20 : H (250) | 18 |
| (100) + CME-(3*Z*,6*Z*,9*Z*)-20 : H (400) | 2 |
| CME-(3*Z*,6*Z*,9*Z*)-21 : H (400) + CME-(3*Z*,6*Z*,9*Z*)-22 : H (100) | 20 |
| (250) + CME-(3*Z*,6*Z*,9*Z*)-22 : H (250) | 9 |
| CME-(3*Z*,6*Z*,9*Z*)-20 : H (500) | 4 |
| CME-(3*Z*,6*Z*,9*Z*)-20 : H (250) + (3*Z*,6*Z*,9*Z*)-20 : H (250) | 5 |

[a] Total number of target males captured in 2× replicated Pherocon 1CP traps. Captures occurred between June 27, and July 25, 1983.

[b] CME represents an equal mixture of the monoepoxydiene hydrocarbons derived from the triene hydrocarbon, here from (3*Z*,6*Z*,9*Z*)-21 : H.

urated $C_{20}$ hydrocarbons were also measured to determine if those with unsaturation corresponding in position and geometry to the field-active compounds would display elevated responses. Near background responses resulted from all. Thus, while some lepidopteran species give elevated EAG responses to monoolefinic analogs of their dienyl acetate, alcohol, or aldehyde pheromones and thus yield information regarding the positions of unsaturation, it would appear this phenomenon does not hold for these polyunsaturated hydrocarbons.

Although fewer specimens of two species of *Idia* were trapped, epoxide isomers were also involved in their capture. A total of 20 specimens of *I. americalis* were taken. Of these, 17 were caught in traps baited with epoxide-based lures and 11 in traps containing CME-(3*Z*,6*Z*,9*Z*)-20:H. The other species, *I. aemula*, was captured only in traps baited with monoepoxydiene hydrocarbons and eight of the 13 specimens were taken in traps containing CME-(3*Z*,6*Z*,9*Z*)-21:H. EAG analyses were not conducted on either of these species.

Traps baited with lures containing CME-(3*Z*,6*Z*,9*Z*)-19:H as the major component captured 10 out of a total of 11 specimens of *Rivula propinqualis* (subfamily Rivulinae), an insect whose range extends from Nova Scotia to Texas and across the continent as far as the Rocky Mountains. None were captured in traps containing either of the singly baited $C_{19}$ (9*R*,10*S*)- or (9*S*,10*R*)-monoepoxydienes, which suggests mixtures of these two enantiomers or another positional epoxide isomer in the CME mixture to be the attractant.

Specific attraction to lures containing primarily CME mixtures was also displayed by several geometrid species. The chevron moth, *Eulithes testata*, which belongs to the same tribe (Hydriomenini) as the *Dysstroma* sp. gave strong EAG signals in response to all of the CME homologs except for the $C_{22}$ homolog (Figure 3). As observed for the *Dysstroma* sp. tentatively identified as *citrata*, all captures of *E. testata* were in traps baited with combinations of (3*Z*,6*Z*,9*Z*)-20:H or (3*Z*,6*Z*,9*Z*)-21:H plus a $C_{20}$ or $C_{21}$ CME or (9*S*,10*R*)-epoxydiene isomer. Although the results do not indicate a specific attractant, responses to the combined monoepoxides may indicate involvement by epoxydiene isomers other than the 9,10-epoxydienes.

Several other species of geometrids were also caught by lures consisting primarily of combined monoepoxydiene mixtures. The EAG profiles for these moths (Figure 5) were similar to each other and showed strong responses to CME-(3*Z*,6*Z*,9*Z*)-trienes and in some cases (3*Z*,6*Z*,9*Z*)-trienes. EAG responses to the (9*S*,10*R*)- and (9*R*,10*S*)-epoxydienes were background or near background for all of these species. In some cases, field captures of these moths also correlated well with their EAG profiles. For example, a *Semiothisa* sp., tentatively identified as *signaria dispuncta* (Walker), gave its strongest antennal response to CME-(3*Z*,6*Z*,9*Z*)-18:H and was captured only by lures containing CME-(3*Z*,6*Z*,9*Z*)-18:H. Captures of eight, seven, seven, and one males were recorded for survey traps baited with CME-(3*Z*,6*Z*,9*Z*)-18:H (500 $\mu$g), CME-(3*Z*,6*Z*,9*Z*)-18:H (400 $\mu$g) + (3*Z*,6*Z*,9*Z*)-18:H (100

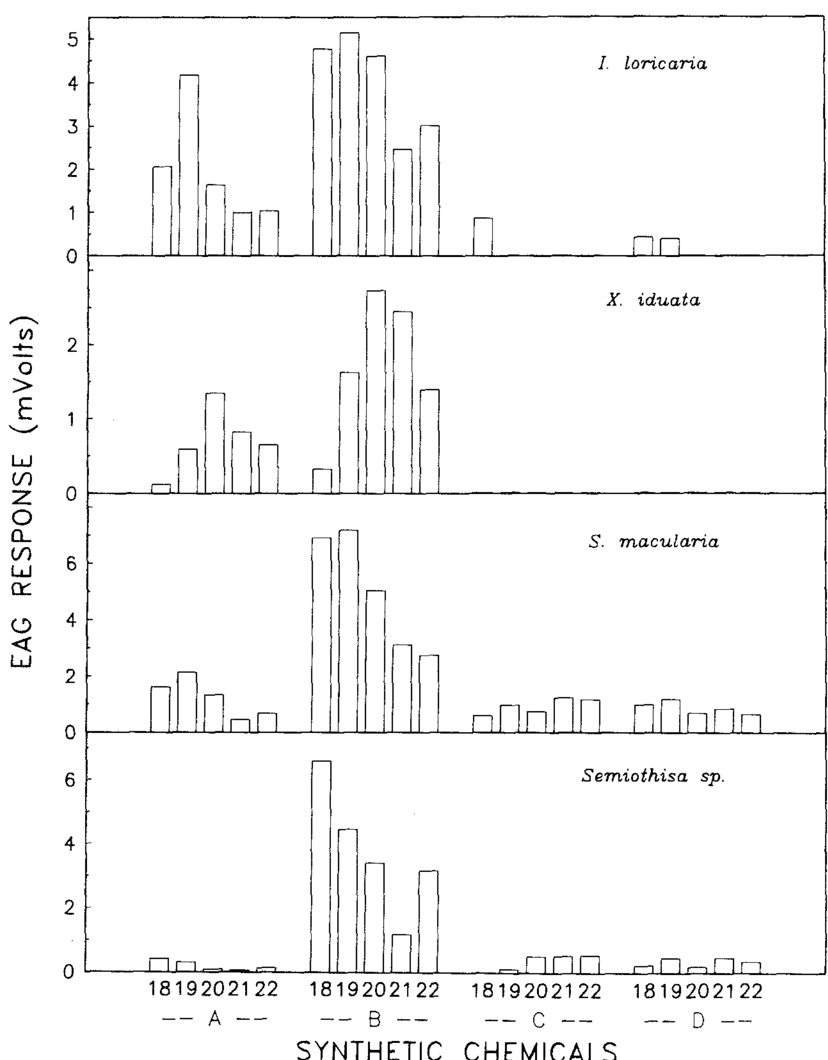


FIG. 5. EAG responses of *I. loricaria, X. iduata, S. macularia*, and a *Semiothisa* sp. Letters (A–D) refer to (3*Z*,6*Z*,9*Z*)-*X*: H, CME-(3*Z*,6*Z*,9*Z*)-*X*: H, (3*Z*,6*Z*)-9*R*,10*S*-epoxy-*X*: H, and (3*Z*,6*Z*)-9*S*,10*R*-epoxy-*X*: H, respectively. Numbers (18–22) refer to the length of the carbon chain (*X*).

$\mu$g), CME-(3*Z*,6*Z*,9*Z*)18 : H (250 $\mu$g) + (3*Z*,6*Z*,9*Z*)-18 : H (250 $\mu$g), and CME-(3*Z*,6*Z*,9*Z*)-18 : H (250 $\mu$g) + CME-(3*Z*,6*Z*,9*Z*)-19 : H (250 $\mu$g), respectively. These captures, together with the absence of captures to (3*Z*,6*Z*)-9*S*,10*R*-epoxy-18 : H or the 9*R*,10*S* isomer, suggest the involvement of (3*Z*,6*Z*)-

*cis*-3,4-epoxy-18:H and/or (3*Z*,6*Z*)-*cis*-6,7-epoxy-18:H in attraction of this species.

Two single captures of *Sicya macularia* were recorded during the 1983 field survey in traps baited with (3*Z*,6*Z*,9*Z*)-20:H (400 μg) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (100 μg) and (3*Z*,6*Z*)-9*R*,10*S*-epoxy-19:H (400 μg) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (100 μg). Although these captures are not meaningful on their own, the EAG profile of *S. macularia* (Figure 5) is very similar to that of the *Semiothisa* sp. and suggests the potential involvement of other monoepoxydiene isomers.

Multiple captures were obtained for three species of *Xanthorhoe* during 1983 and are shown in Table 10. All three species responded to lures consisting primarily of combined monoepoxydienes with exceptions of single captures of *X. abrasaria aquilonaria* and *X. munitata*. Again there was good correlation between field captures and EAG responses as exemplified by *X. iduata* (Figure 5). The EAG profile of *X. munitata* (not shown) is virtually identical to that of *X. iduata* with strongest responses recorded for the $C_{20}$ and $C_{21}$ homologs of CME-(3*Z*,6*Z*,9*Z*)-trienes. An EAG profile was not recorded for *X. abrasaria aquilonaria*.

The EAG profile of *Itame loricaria* (Eversmann) (Figure 5) is similar to the others in the figure except that responses are somewhat higher for (3Z,6Z,9Z)-trienes. Single captures of *I. loricaria* were obtained for 10 different lures consisting of (3Z,6Z,9Z)-trienes, combined monoepoxydienes, and specific epoxydiene enantiomers. A specific attractant was not discerned from these results. Unlike *Xanthorhoe* sp. and *Semiothisa* sp., whose field captures were predicted by their EAG profiles, the failure of field captures of *I. loricaria* to correspond to its EAG profile may indicate both attraction and inhibition by different epoxydiene isomers within the combined monoepoxydiene mixtures.

Multiple captures of *Eupithecia annulata* were recorded for several lures during 1983. While an EAG profile was not recorded, field captures (Table 11) indicated that *E. annulata* may belong to the group of moths that respond to epoxydienes other than the 9,10 isomers. However, the lack of response to the (9*R*,10*S*)- and (9*S*,10*R*)-epoxydienes does not rule out the possibility that a specific combination of the two enantiomers is responsible for attraction. CME-(3Z,6Z,9Z)-20:H appears to be the strongest attractant and was not potentiated by other compounds tested in the survey.

Three single captures each of *E. rovocastaliata* (Packard) and *E. satyrata dodata* (Taylor) were recorded in the survey. All of the lures consisted of a (3Z,6Z,9Z)-triene ($C_{19}$ or $C_{21}$) plus a specific epoxide enantiomer or CME mixture of the same chain length.

## DISCUSSION

Our choice of potential attractants for field screening was based on the structures of pheromones for two noctuid species and several arctiid species.

TABLE 10. CAPTURE OF *Xanthorhoe abrasaria aquilonaria*, *X. iduata*, AND *X. munitata* IN SURVEY TRAPS

| Lure composition (μg) | Total males captured | | |
|---|---|---|---|
| | *X. a. aquilonaria*[a] | *X. iduata*[b] | *X. munitata*[c] |
| CME-(3*Z*,6*Z*,9*Z*)-21:H (100) + (3*Z*,6*Z*,9*Z*)-21:H (400) | 5 | 7 | 0 |
| (250) + (3*Z*,6*Z*,9*Z*)-21:H (250) | 1 | 8 | 0 |
| (400) + (3*Z*,6*Z*,9*Z*)-21:H (100) | 0 | 1 | 2 |
| (3*Z*,6*Z*,9*Z*)-19:H (250) + (3*Z*,6*Z*,9*Z*)-20:H (250) | 1 | 0 | 0 |
| CME-(3*Z*,6*Z*,9*Z*)-21:H (500) | 0 | 0 | 3 |
| (400) + CME-(3*Z*,6*Z*,9*Z*)-20:H (100) | 0 | 0 | 1 |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21:H (500) | 0 | 0 | 2 |

[a] Total males captured in 2X replicated Pherocon 1CP traps from July 18 to August 4, 1983.
[b] Total males captured in 2X replicated Pherocon 1CP traps from July 4 to July 28, 1983.
[c] Total males captured in 2X replicated Pherocon 1CP traps from May 25 to June 3, 1983.

TABLE 11. CAPTURE OF *Eupithecia annulata* IN SURVEY TRAPS

| Lure composition ($\mu$g) | Total males captured[a] |
|---|---|
| CME-(3*Z*,6*Z*,9*Z*)-18:H (400) + CME-(3*Z*,6*Z*,9*Z*)-19:H (100) | 2 |
| (100) + CME-(3*Z*,6*Z*,9*Z*)-19:H (400) | 3 |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-18:H (500) | 1 |
| CME-(3*Z*,6*Z*,9*Z*)-19:H (500) | 2 |
| (400) + (3*Z*,6*Z*,9*Z*)-19:H (100) | 2 |
| (400) + CME-(3*Z*,6*Z*,9*Z*)-20:H (100) | 8 |
| (250) + CME-(3*Z*,6*Z*,9*Z*)-20:H (250) | 9 |
| (100) + CME-(3*Z*,6*Z*,9*Z*)-20:H (400) | 17 |
| CME-(3*Z*,6*Z*,9*Z*)-20:H (500) | 16 |
| (400) + CME-(3*Z*,6*Z*,9*Z*)-21:H (100) | 5 |
| (250) + CME-(3*Z*,6*Z*,9*Z*)-21:H (250) | 4 |
| (100) + CME-(3*Z*,6*Z*,9*Z*)-21:H (400) | 1 |
| CME-(3*Z*,6*Z*,9*Z*)-21:H (400) + CME-(3*Z*,6*Z*,9*Z*)-22:H (100) | 2 |
| (3*Z*,6*Z*)-9*R*,10*S*-epoxy-21:H (400) + (3*Z*,6*Z*)-9*R*,10*S*-epoxy-20:H (100) | 1 |
| (3*Z*,6*Z*)-9*S*,10*R*-epoxy-19:H (250) + (3*Z*,6*Z*,9*Z*)-19:H (250) | 1 |

[a]Total males captured in 2× replicated Pherocon 1CP traps from May 16 to June 30, 1983.

The discovery of (3Z,6Z,9Z)-21:H as a pheromone component for *Utetheisa ornatrix* (Conner et al., 1980), and the discovery of this compound together with (3Z,6Z,9Z)-20:H as pheromone components for *Anticarsia gemmatalis* (Hübner) (Heath et al., 1983) and *Caenurgina erechtea* (Underhill et al., 1983), revealed the methylene interrupted triene moiety which had not appeared in previously reported lepidopteran pheromones. The discovery of (3Z,6Z)-*cis*-9,10-epoxy-21:H as a pheromone component for the saltmarsh caterpillar moth, *Estigmene acrea* (Drury) (Hill and Roelofs, 1981), and the fall webworm moth, *Hyphantrea cunea* (Drury) (Hill et al., 1982) indicated the potential importance of monoepoxidized derivatives of the (3Z,6Z,9Z)-trienes as lepidopteran attractants.

Field screening of (3Z,6Z,9Z)-triene hydrocarbons and monoepoxydiene derivatives has resulted in the discovery of specific attractants for males of several species of geometrids from the two major subfamilies of Ennominae and Larentiinae. The specificity of response by captured species was very high, and for some species, *M. inatomaria*, *C. erythemaria*, *P. transversata*, and *D. brunneata ethela*, the ability to discriminate between opposite enantiomers or a particular ratio of enantiomers was clearly demonstrated by field captures and EAG responses. Although many examples of chiral insect pheromones are known (Brand et al., 1979), only two examples have been clearly identified for lepidopteran species. These are *cis*-7,8-epoxy-2-methyloctadecane (disparlure) from

the gypsy moth (Bierl et al., 1970), and (*R*)-1-methybutyl decanoate from the bagworm moth, *Thyridopteryx ephemeraeformis* (Haworth) (Leonhardt et al., 1983). Some evidence for the involvement of chirality in the pheromone system of *E. acrea* and *H. cunea* has been reported (Hill and Roelofs, 1981; Hill et al., 1982). Tamaki et al. (1983) have also reported a chiral pheromone component, 10-methyldodecyl acetate, for the smaller tea tortrix moth. The results of our field screening, performed with compounds which have been identified as pheromone or homologs of pheromone, suggest a wider involvement of chirality in lepidopteran pheromone systems.

The data presented here and elsewhere (Heath et al., 1983; Underhill et al., 1983) also expands the structural group of chemicals associated with male noctuid sex attraction to include (3Z,6Z,9Z)-triene hydrocarbons and a number of their monoepoxydiene hydrocarbon analogs. Although only a few species were captured, our survey has increased the number of noctuid subfamilies linked to defined chemical attractants from nine to 13. Further field tests to improve lure efficiency and further define optimal chemical attractants for these noctuid and geometrid species are planned. On the basis of the data presented here, we believe that unsaturated hydrocarbons and monoepoxydiene derivatives may function as pheromones for some geometrid and noctuid species.

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# FIELD RESPONSE OF *Dendroctonus brevicomis*[1] TO *exo*-BREVICOMIN, FRONTALIN, AND MYRCENE RELEASED AT TWO PROPORTIONS AND THREE LEVELS[2]

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**Abstract**—Racemic *exo*-brevicomin, racemic frontalin, and myrcene were released at two proportions (5:1:400 and 1:1:1), each at three levels (1×, 10×, and 100×) in a ponderosa pine forest in central California. The 5:1:400 mix was based on an estimate of the relative amounts released from a ponderosa pine under attack by the western pine beetle, *Dendroctonus brevicomis*. More *D. brevicomis* were trapped at a source of the three compounds released at 5:1:400 than were trapped at a source released at 1:1:1, at all three levels, but this difference was statistically significant only at the 1 × and 10 × levels. Sex ratio of trapped beetles and distribution of catch at the source of attractant and 5 m away apparently did not differ between relative release rates.

**Key Words**—*Dendroctonus brevicomis*, Coleptera, Scolytidae, bark beetle, western pine beetle, attractant rate, pheromone, *exo*-brevicomin, frontalin, myrcene.

## INTRODUCTION

The western pine beetle, *Dendroctonus brevicomis* LeConte, was attracted in flight to the mixture of *exo*-brevicomin (E) (Silverstein et al., 1968), frontalin (F) (Kinzer et al., 1969), and myrcene (M) (Bedard et al., 1969; Silverstein, 1970). Mixtures containing (1*R*,5*S*,7*R*)-(+)-*exo*-brevicomin and (1*S*,5*R*)-(−)-frontalin were more attractive than mixtures containing the antipodes or racemic compounds (Wood et al., 1976). Female *D. brevicomis* boring in bolts of pon-

[1] Coleoptera: Scolytidae.
[2] Trade names are mentioned solely for information. No endorsement by the U.S. Department of Agriculture is implied.

derosa pine, *Pinus ponderosa* Dougl. ex Laws., released (+)E, and hindguts of males contained (−)F (Stewart et al., 1977). Most studies of the response of *D. brevicomis* to EFM, however, have used racemic E and F, owing to the unavailability of pure enantiomers (Bedard and Wood, 1974, 1981; Bedard et al., 1969, 1980; Tilden et al., 1979, 1981, 1983).

In the above-mentioned studies, the ratio of E:F:M released was 1:1:1. Browne et al. (1979) measured amounts of EFM released from ponderosa pine bolts infested with *D. brevicomis* in the laboratory and from standing trees attacked in the field, during periods of 12–48 hr. They estimated the relative amounts of EFM released by an equal number of male and female *D. brevicomis* attacking ponderosa pine would be about 5:1:400. Although the enantiomeric composition of E and F was not determined, we assume that the compounds were (+)E and (−)F (Stewart et al., 1977).

This paper reports a study of the numbers of *D. brevicomis* trapped at and near sources of EFM, to determine whether the estimated 5:1:400 proportion would attract different numbers of western pine beetles than did the 1:1:1 proportion used in earlier studies. This information could be used to specify baits for survey and suppression traps and to design future behavioral studies of response to pheromones. Three levels of attractant were tested because the numbers of *D. brevicomis* trapped varied with attractant release rate (Tilden et al., 1983).

## METHODS AND MATERIALS

The study was done on the Sierra National Forest, near Oakhurst, California, from July 21 to August 20, 1981. Six plots were established in 1-hectare or larger openings in ponderosa pine forest (1100–1340 m elevation), with at least 1.6 km between plots. Five 25.4-cm-diam. × 3-m Sonotube® cardboard cylinders were erected vertically in each plot to simulate tree trunks (Tilden et al., 1983). One cylinder was placed at the center of a circle with a 5-m radius, and the other four cylinders were placed 90 degrees apart on the circumference of the circle. A cylindrical hardware cloth (6.35-mm mesh) trap, 30.5 cm high × 34 cm diam. and coated with melted Stikem Special®, was hung around each cardboard cylinder, with the center of the trap 1.5 m above ground.

Racemic E, racemic F, and natural M (purity >98%) were evaporated at the center cardboard cylinder 1.5 m above ground, at two proportions and three levels in three treatment pairs: 5:1:400 vs 1:1:1 (E:F:M) at 1×, 10×, and 100× levels. Each of the three treatment pairs was arranged in a cross-over design (Cochran and Cox, 1957), with each pair randomly assigned to two plots. Each member of a pair was randomly assigned to eight days per plot, for a total of 16 days (replicates) per treatment. For example, at the 1× level 5:1:400 and 1:1:1 (treatments A and B) were assigned to plots 1 and 2 for 16 days as follows:

Plot 1: A B B A B A B A B A B A B A B A

Plot 2: B A A B A B A B A B A B A B A B

The total amount of E + F released at 1:1 and 5:1 was about the same at each level. At the 1× level, for example, the total amounts of E + F released per 24 hr were 0.81 mg (1:1) and 0.84 mg (5:1) (Table 2). As was the practice in some earlier studies, we used racemic E and F because the pure enantiomers were not available. The presence of unattractive enantiomers evidently does not interrupt response (Wood et al., 1976).

The following devices were used to release the compounds: for E or M—glass pipets, 0.80 mm ID × 50 mm, glass tubes, 3.5 mm ID × 52 mm, and 1-dr glass vials, 9 mm ID × 45 mm; for M only—plastic cups, 32 mm ID × 15 mm; and for F—glass pipets, 0.40 mm ID × 32 mm, glass tubes, 2.2 mm ID × 62 mm, and $\frac{1}{2}$-dr glass vials, 5 mm ID × 35 mm (Tilden et al., 1983). Glass pipets and tubes were flame-sealed at one end. Devices were put in containers to provide protection from wind and sun and were hung from a stand adjacent to the center cardboard cylinder.

At the 1× level, the 1:1:1 treatment was three pipets of each compound inside a perforated aluminum tea ball, 5 cm maximum ID × 6 cm. The 5:1:400 treatment was five pipets of E and one pipet of F inside a tea ball, and three vials of M inside an inverted glass jar, 4 cm ID × 6.4 cm, covered with aluminum foil and with punctured lid.

At the 10× level, the 1:1:1 treatment was three glass tubes of each compound inside two inverted salt shakers covered with aluminum foil. The 5:1:400 treatment was five tubes of E, one tube of F, and 37 vials of M inside a cylindrical hardware cloth container, 12.7 cm diam. × 15.2 cm, covered with aluminum foil and with removable aluminum lid. The tubes and vials were set on a hardware cloth shelf inside the container.

At the 100× level, the 1:1:1 treatment was three vials of E, two vials of F, and two vials of M inside two inverted glass jars (described above). The 5:1:400 treatment was five vials of E, eight tubes of F, and 16 plastic cups of M inside a coffee can, 15.2 cm diam. × 17.8 cm. The bottom of the can was perforated by drilling 3.18-mm-diam. holes. The devices were set on a hardware cloth shelf midway in the can, which was covered with an aluminum lid.

Before the start of the test, the release rates of the compounds were measured gravimetrically. Devices were exposed inside a standard weather shelter (Fischer and Hardy, 1976) near the Oakhurst Ranger Station (790 m elevation), where temperatures were assumed to be similar to temperatures in the plots 5–10 km away. Five groups of three glass pipets of each compound were measured after three 72-hr periods, and five groups of three pipets each were measured after four 24-hr periods. Five glass tubes of each compound were measured after seven 24-hr periods. Five plastic cups of M were measured after two 24-hr periods. Means were calculated and variance components due to devices,

days, and unexplained error were estimated by analysis of variance of the weight loss data. Weight loss of the devices was not measured during the test.

Compounds were put in the plots between 1300 and 1500 hr, before peak beetle flight, and picked up the next morning between 0700 and 0830 hr. *D. brevicomis* were then picked from all traps and placed in labeled vials of kerosene for later counting and sex determination.

Catches at the center trap and the combination of the four outlying traps were examined by analysis of variance with the daily catches untransformed ($x$) and transformed by $\log_e (x + 1)$ to equalize variances. The error mean square from the analysis of variance of the untransformed data was used as an estimate of the common variance for each treatment pair and to calculate 95% confidence intervals for differences in treatment means.

## RESULTS

Weight loss of release devices measured before the start of the test (Table 1) was used to determine the number and type of devices for each treatment and to predict the amounts of EFM released (Table 2). Assuming the release rates

TABLE 1. MEAN WEIGHT LOSS OF RACEMIC *exo*-BREVICOMIN, RACEMIC FRONTALIN, AND MYCRENE FROM RELEASE DEVICES NEAR OAKHURST, CALIFORNIA, JUNE 3–JULY 14, 1981

| Compound and device[a] | Weight loss (mg/24 hr)[b] | Variance due to | | |
|---|---|---|---|---|
| | | Day | Device | Error |
| *exo*-Brevicomin | | | | |
| Pipet | 0.14 (0.07) | 66 | 7 | 27 |
| Tube | 1.40 (0.23) | 77 | 8 | 15 |
| Vial | 14.27 (1.36) | 79 | 0 | 21 |
| Frontalin | | | | |
| Pipet | 0.13 (0.08) | 42 | 0 | 58 |
| Tube | 1.58 (0.25) | 51 | 27 | 22 |
| Vial | 21.97 (1.13) | 77 | 15 | 8 |
| Myrcene | | | | |
| Pipet | 0.16 (0.08) | 48 | 0 | 52 |
| Tube | 1.86 (0.27) | 73 | 14 | 13 |
| Vial | 18.93 (1.17) | 72 | 4 | 24 |
| Cup | 368.74 (16.73) | 78 | 13 | 9 |

[a]See text for description of devices.
[b]Mean (standard deviation) per device.

TABLE 2. MEAN RELEASE RATES (mg/24 hr) OF RACEMIC *exo*-BREVICOMIN (E), RACEMIC FRONTALIN (F), AND MYRCENE (M), PREDICTED FROM MEASURED WEIGHT LOSS OF RELEASE DEVICES[a]

| Proportion[b] and level | *exo*-Brevicomin | Frontalin | Myrcene |
|---|---|---|---|
| 1:1:1 | | | |
| 1x | 0.43 (0.19)[c] | 0.38 (0.18) | 0.48 (0.19) |
| 10x | 4.19 (0.63) | 4.75 (0.63) | 5.58 (0.73) |
| 100x | 42.82 (3.78) | 43.93 (2.11) | 37.85 (2.17) |
| 5:1:400 | | | |
| 1x | 0.71 (0.30) | 0.13 (0.08) | 56.78 (3.16) |
| 10x | 6.99 (1.03) | 1.58 (0.25) | 700.30 (36.87) |
| 100x | 71.37 (6.20) | 12.66 (1.54) | 5899.84 (238.15) |

[a]See text for description and number of devices used for each treatment.
[b]E:F:M.
[c]Standard deviations are in parentheses.

of (+)E and (−)F were about half that of the racemic compounds, the actual proportions of (+)E, (−)F, and M were about 1:1:2 and 5:1:800, but the labels 1:1:1 and 5:1:400 are used to be consistent with earlier studies. Analysis of variance of the weight loss measurements showed that 42–79% of the variation in release rate of one device in a 24 hr period was due to day effects (Table 1).

Analysis of variance of the untransformed and transformed daily catches of *D. brevicomis* gave essentially the same patterns of significant $F$ ratios, so only the analysis of the untransformed data is presented. Catch at the center trap varied significantly ($p < 0.01$) between treatments at the 1× and 10× levels (Table 3). At the four outlying traps, catch varied significantly ($p < 0.05$) between treatments at the 1× level only. At the 10× treatment level, plots were a source of statistically significant ($p < 0.01$) variation for catch at the center and outlying traps. At the 100× treatment level, days and plots were sources of statistically significant ($p < 0.01$) variation for catch at the center trap (Table 3).

The differences in mean catch between treatments (catch at 5:1:400 less catch at 1:1:1, Table 4) and corresponding 95% confidence intervals were as follows:

| Level | Center trap | Four outlying traps |
|---|---|---|
| 1× | 13.6 ± 9.4 | 1.2 ± 1.0 |
| 10× | 41.7 ± 27.5 | 7.1 ± 7.9 |
| 100× | 31.4 ± 31.7 | 1.5 ± 15.9 |

TABLE 3. ANALYSIS OF VARIANCE OF *Dendroctonus brevicomis* CAUGHT AT SOURCE OF RACEMIC *exo*-BREVICOMIN (E), RACEMIC FRONTALIN (F), AND MYCRENE (M) RELEASED AT TWO PROPORTIONS[a] AND THREE LEVELS, AND AT FOUR TRAPS 5 m AWAY FROM SOURCE, NEAR OAKHURST, CALIFORNIA, JULY 21–AUGUST 20, 1981

| | | Catch at | | | |
|---|---|---|---|---|---|
| | | Center trap | | 4 Outlying traps | |
| Source of variations | Degrees of freedom | Mean square | $F^b$ | Mean square | $F^b$ |
| Treatment level: 1x | | | | | |
| Days | 15 | 281 | 1.83 | 2.6 | 1.62 |
| Plots | 1 | 545 | 3.55 | 3.1 | 1.96 |
| Treatments | 1 | 1485 | 9.67** | 12.5 | 7.81* |
| Error | 14 | 154 | | 1.6 | |
| Total | 31 | | | | |
| Treatment level: 10x | | | | | |
| Days | 15 | 2857 | 2.17 | 79 | 0.73 |
| Plots | 1 | 16110 | 12.23** | 1313 | 12.19** |
| Treatments | 1 | 13861 | 10.52** | 399 | 3.70 |
| Error | 14 | 1318 | | 108 | |
| Total | 31 | | | | |
| Treatment level: 100x | | | | | |
| Days | 15 | 10974 | 6.30** | 663 | 1.55 |
| Plots | 1 | 25313 | 14.53** | 1800 | 4.21 |
| Treatments | 1 | 7875 | 4.52 | 18 | 0.04 |
| Error | 14 | 1742 | | 428 | |
| Total | 31 | | | | |

[a] 1 : 1 : 1 and 5 : 1 : 400 E : F : M. See Table 2 for predicted release rates.
[b] Statistically significant $F$ ratios indicated by * ($p < 0.05$) or ** ($p < 0.01$).

More *D. brevicomis* were caught at the center trap at the 5:1:400 treatments than at the 1:1:1 treatments, but this difference at the 100× level was not statistically significant, as the confidence interval for the difference in treatment means included zero. More *D. brevicomis* were trapped at the outlying traps at the 5:1:400 treatments than at the 1:1:1 treatments, but these differences were not statistically significant at the 10× and 100× levels. At each treatment level, the male proportions of beetles caught at the center or outlying traps (Table 4) apparently did not differ between treatments.

We could not make statistically valid comparisons between the three levels (1×, 10×, 100×) because each treatment pair appeared in only two of the six

TABLE 4. CATCH OF *Dendroctonus brevicomis* AT SOURCE OF RACEMIC *exo*-BREVICOMIN (E), RACEMIC FRONTALIN (F), AND MYRCENE (M) RELEASED AT TWO PROPORTIONS AND THREE LEVELS, AND AT FOUR TRAPS 5 m AWAY FROM SOURCE, NEAR OAKHURST, CALIFORNIA, JULY 21–AUGUST 20, 1981

| Treatment level and proportion[a] | *D. brevicomis* caught at | | | | |
|---|---|---|---|---|---|
| | Center Trap | | | 4 Outlying Traps | |
| | % at center | $\bar{X}$ (SD)[b] | Male prop.[c] | $\bar{X}$ (SD)[b] | Male prop.[c] |
| 1x | | | | | |
| 1:1:1 | 95.2 | 11.8 (12.4) | 0.612 | 0.6 (1.3) | 0.667 |
| 5:1:400 | 93.4 | 25.4 (12.4) | 0.613 | 1.8 (1.3) | 0.655 |
| 10x | | | | | |
| 1:1:1 | 82.6 | 87.6 (36.3) | 0.558 | 18.5 (10.4) | 0.510 |
| 5:1:400 | 83.5 | 129.3 (36.3) | 0.558 | 25.6 (10.4) | 0.597 |
| 100x | | | | | |
| 1:1:1 | 76.3 | 110.8 (41.7) | 0.541 | 34.5 (20.7) | 0.578 |
| 5:1:400 | 79.8 | 142.2 (41.7) | 0.553 | 36.0 (20.7) | 0.569 |

[a]E:F:M. See Table 2 for predicted release rates.
[b]Mean catch at one trap (center) or at total of four traps (outlying) per day, $N = 16$. Number in parentheses is one standard deviation, calculated from error mean square (Table 3), an estimate of the common variance.
[c]Male proportion of catch.

plots, and we had no estimate of the variance between the three pairs of plots. Examination of the data (Table 4), however, shows that the numbers of beetles' caught at the center and outlying traps increased as the attractant level increased. Also, the percentage caught at the center trap decreased and the percentage caught at the outlying traps increased as the attractant level increased.

## DISCUSSION

Catch of *D. brevicomis* at and near a source of EFM at 1:1:1 was related to trap size (Tilden et al., 1979), presence of a tree trunk silhouette, and attractant release rate (Tilden et al., 1983). Although we did not compare EFM at 5:1:400 with a natural source of attractant, we assume that this mixture is more like that source than is EFM at 1:1:1 (Browne et al., 1979). If this is true, our results validate the above-mentioned conclusions of previous studies of EFM at 1:1:1, because the only apparent difference in the response of *D.*

*brevicomis* to EFM at 1:1:1 and 5:1:400 at each treatment level was in the numbers of beetles trapped. Sex ratio and distribution of trapped beetles apparently did not differ. Within at least this range in proportions, there may be some leeway in specifying an attractant for survey and suppression traps, as well as for further research, without drastically affecting the behavior of *D. brevicomis* in response to a source of attractant.

Bedard et al. (unpublished) found that increasing the release rates of 1:1:1 EFM 10-fold and 100-fold not only increased the total number of *D. brevicomis* trapped, but also decreased the proportion of beetles trapped at the source of attractant and increased the proportions trapped 4.5 and 9 m away. The numbers and distribution of the southern pine beetle, *D. frontalis* Zimm., trapped at and up to 10 m away from a source of attractant released at several rates followed a similar pattern (Vité, 1970). In our study, 10-fold and 100-fold increases in the release rates of EFM at 1:1:1 or 5:1:400 apparently had similar effects (Table 4), but they could not be assessed statistically.

While we did not attempt to examine effects of various relative amounts of each compound on beetle response, the differences in catch at each treatment level we observed could be due to changes in the two beetle-produced compounds E and F relative to each other, or to increased amounts of the host compound M, or both. In a previous study, the number of *D. brevicomis* trapped decreased when the release rate of M was reduced 10-fold relative to E and F (10:10:10 vs. 10:10:1 of E:F:M), with about 2 mg/24 hr per compound released at the 1× level (Bedard et al., unpublished). Increasing the amount of one beetle-produced component of the pheromone of *Scolytus multistriatus* (Marsham) (Coleoptera: Scolytidae) relative to two other components (one from the beetle and one from the host tree) decreased the number of beetles trapped, and increasing the amount of the host-tree component relative to the beetle-produced components increased the number of beetles trapped (Cuthbert and Peacock, 1978). In both these studies, as in ours, manipulating the release rate of the host component of the pheromone affected the numbers of beetles trapped. From the standpoint of beetles' use of energy and resources, it may be more efficient to increase aggregation on a potential host by boring activity, which releases large amounts of host compounds, than by releasing large amounts of beetle-produced compounds, e.g., E and F.

The variation in release rate of compounds due to variation among days noted during the preliminary measurements of release devices was probably due largely to temperature. While the measured devices were exposed inside a weather shelter at one location, devices in the test plots were exposed to both elevation and temperature differences. Variation in release rate due to day, plot, and device effects—as well as unexplained causes—may contribute to variation in numbers of beetles trapped, as do day and plot variations in numbers of beetles available to respond to a source of attractant.

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# ELECTROANTENNOGRAM RESPONSES OF *Trirhabda bacharides* (WEBER) (COLEOPTERA: CHRYSOMELIDAE) TO PLANT VOLATILES[1]

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**Abstract**—Electroantennograms (EAGs) were recorded from *Trirhabda bacharides* Le Conte (Coleoptera: Chrysomelidae) females to 28 odorants and volatiles emanating from leaves of 26 plant species including three known host plants, *Baccharis halimifolia* L., *B. neglecta* Britt., and *B. salicina* T. & G. (Compositae: Asterae). Antennal receptors were highly responsive to components of the green leaf volatile complex, especially 6-carbon saturated and monounsaturated alcohols and *trans*-2-hexenal. EAGs elicited by heptanal were greater than those elicited by any other saturated aldehyde. Oxygenated monoterpenes were more active than monoterpene hydrocarbons. β-Bisabolol was the most active sesquiterpene. In general, EAGs to volatiles emanating from *Baccharis* and other composite species were greater than those elicited by species from other families. Furthermore, when one considers only plants occurring in the environs of *T bacharides* host *B. neglecta*, EAGs elicited by its host plant were at least two times greater than those elicited by other coinhabiting plants.

**Key Words**—Host plant, biological control of weeds, *Trirhabda bacharides*, Coleoptera, Chrysomelidae, Compositae, *Baccharis*, green leaf volatiles, plant odor, electroantennogram, olfaction.

## INTRODUCTION

The perennial woody shrubs, *Baccharis halimifolia* L. and *B. neglecta* Britt. (Compositae: Astereae) are undesirable weeds, with little or no value to agri-

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

culture or wildlife (Tarver et al., 1979). *B. halimifolia* is abundant in wet low plains, marshes, and swamps from Massachusetts to Florida and west to Texas (Tarver et al. 1979). Its leaves and flowers contain cardiac glycosides which can cause staggering, trembling, convulsions, diarrhea, and other gastrointestinal symptoms in sheep and cattle (Cooperative Extension Service, 1980). *B. neglecta* is an increasing weed problem on native rangeland and pasture in Texas (Mutz et al., 1979). Over 201,000 acres of rangeland were infested in 1973, and the area infested grew significantly from 1973 to 1983 in central and southern Texas (SCS Tex. records).

Members of the genus *Trirhabda* LeConte (Coleoptera: Chrysomelidae) are oligophagous on the leaves and inflorescences of certain Compositae and Hydrophyllaceae (Hogue, 1970). One of the 24 species in the genus, *T. bacharides* (Weber) is the dominant herbivore on *B. halimifolia* (Hasler, 1969; Kraft and Denno, 1982) and recently was observed to feed in large numbers on *B. neglecta* in Texas (DeLoach and Boldt, unpublished). *T. bacharides* is univoltine, although a partial second generation was reported in Florida by Bennett (1963). Larvae emerge in the spring from overwintered eggs laid in cracks on the bark and feed on young leaves of the plant. Pupation occurs in the soil. Adults emerge in late spring and summer and continue to feed on the leaves (Hogue, 1970). Damage is severe, and both leaves and bark are often stripped from the plant by the adults.

In multiple-choice feeding tests with 67 species of plants representing 23 families and including five species of Compositae, feeding of adult beetles was observed only on the control plant, *B. halimifolia* (Hasler, 1969). Other feeding tests, by Kraft and Denno (1982), indicated that larvae preferred young over old *Baccharis* leaves and could tolerate high concentrations of acetone-soluble chemicals from leaves of *B. halimifolia*. Adult beetles are strong fliers and are able to find and colonize isolated and recently established shrubs. Therefore it is hoped that *T. bacharides* may be used as a biological control agent for *B. halimifolia*.

The purpose of this study was to elucidate the antennal olfactory responsiveness of *T. bacharides* to plant volatiles and other selected odorants and to investigate the responsiveness of its antennal receptors to volatiles emanating from known host plants and other plant species. It is also intended that this study provide a base for future studies of the chemistry of *Baccharis* sp. and *Trirhabda–Baccharis* interactions.

## METHODS AND MATERIALS

*T. bacharides* adults were collected in May 1983, from a dense stand of *B. neglecta* (2–3 m height) growing in abandoned loamy sand soil at the Stillhouse Hollow Dam, 8 km SW of Belton, Texas. They were transported within a few

days to the USDA Boll Weevil Research Laboratory, Mississippi State, Mississippi, in a precooled insulated chest which maintained temperatures between 5 and 10°C.

Methodology for recording electroantennograms (EAGs) is described in detail elsewhere (Dickens, 1979, 1984) and was modified from earlier techniques (Schneider, 1957). Briefly stated, Ag–AgCl capillary electrodes filled with physiological saline (Pantin, 1948; Oakley and Schafer, 1978) were used. Following prepuncture with a sharpened tungsten needle, the recording electrode was inserted into the distal end of the terminal antennal segment; the indifferent electrode was inserted into the scape. The signal was amplified 10-fold by a Grass P-16 differential microelectrode preamplifier and viewed on a Tektronix 5223 digitizing oscilloscope. EAGs were recorded for subsequent analyses and storage using an *X–Y* plotter.

Since the chemistry of *B. neglecta* has not yet been investigated, volatiles used as olfactory stimuli were chosen based primarily on their presence in related Compositae (Uchio et al., 1981; MacLeod et al., 1982; Harada and Iwasaki, 1982) and/or in the green leaf volatile complex (Visser et al., 1979). Several isomers of these compounds as well as other related compounds were also included. Chemicals used in these studies are listed in Table 1.

In order to elucidate the selectivity of the antennal receptors for the various odorants, EAGs were recorded to a 100-μg dose of each. Nanograde pentane dilutions of each compound at 10 μg/μl solvent were delivered as 10-μl samples (i.e., 100 μg stimulus) placed on filter paper (8 × 18 mm) inserted into glass cartridges (80 mm long × 5 mm ID) and oriented toward the preparation from ca. 1 cm. Hydrocarbon-free air (filtered and dried) carried odor molecules evaporating from the filter paper over the antennal preparation. Stimulus duration was 1 sec at 1 m/sec as measured by a thermistor. The atmosphere around the preparation was continuously exhausted. Since the various odorants tested had differing volatilities, comparisons made between odorous stimuli are relative. EAGs from three female beetles were recorded to each stimulus with at least 4 min between each stimulation. This time allowed for complete recovery of the EAG.

In a second experiment, EAGs were recorded from a *T. bacharides* female to volatiles emanating from its known host plants, *B. halimifolia* and *B. neglecta*, and 24 other plant species including 16 Compositae. To evaluate the responsiveness of antennal receptors to each plant species, segments of mature leaves (5 × 10 mm) were crushed between a folded piece of filter paper (8 × 19 mm). Volatiles emanating from the crushed leaf were used as olfactory stimuli in a manner similar to that utilized in the initial experiment with the individual chemicals.

In order that responses from different preparations could be compared, 1-hexanol at 100-μg was used as a standard. Stimulation with the standard either preceded or followed each stimulus by 4 min. Reponses to intervening test stim-

TABLE 1. SOURCE AND PURITY OF CHEMICALS USED IN ELECTROPHYSIOLOGICAL STUDIES AND THEIR PRESENCE IN RELATED COMPOSITAE.

| Compound | Chemical purity (%) | Source[a] | Presence in related compositae[b] |
|---|---|---|---|
| Aliphatic alcohols | | | |
| 1-Butanol | >99 | A | |
| 1-Pentanol | >99 | B | |
| 1-Hexanol | 98 | C | |
| *trans*-2-Hexen-1-ol | 97 | C | + |
| *cis*-3-Hexen-1-ol | 98 | C | + |
| 1-Heptanol | 98 | C | |
| 1-Octanol | 99+ | C | |
| 1-Nonanol | 97 | C | |
| Aliphatic aldehydes | | | |
| Hexanal | 99 | C | + |
| *trans*-2-Hexanal | 99 | C | + |
| Heptanal | 95 | C | |
| Octanal | 99 | C | |
| Nonanal | 98 | C | |
| Decanal | 98 | C | |
| Monoterpene hydrocarbons | | | |
| (−)-Limonene | 97 | C | + |
| (+)-Limonene | 97 | C | + |
| Myrcene | 85 | C | + |
| (−)-α-Pinene | 98 | C | + |
| (+)-α-Pinene | 98 | C | + |
| (−)-β-Pinene | 98 | C | + |
| Oxygenated monoterpenes | | | |
| d-Carvone | 96 | C | |
| *l*-Carvone | 98 | C | |
| Linalool | 99 | C | + |
| Sesquiterpenes | | | |
| β-Bisabolol | 92 | D | |
| Caryophyllene oxide | 97 | C | |
| β-Caryophyllene | 90.2 | E | + |
| Other compounds | | | |
| 1, 8-Cineole | 99 | C | + |
| 2-Phenoxy-ethanol | 99 | C | |

[a] A, Fisher Scientific Co., Pittsburg, Pennsylvania; B, J. T. Baker Chem. Co., Phillipsburg, New Jersey; C, Aldrich Chem. Co., Milwaukee, Wisconsin; D, P. A. Hedin, USDA, ARS, Mississippi State, Mississippi; E, ICN-K&K Laboratories, Inc., Plainview, New York.
[b] Harada and Iwasaki (1982); MacLeod et al. (1982); Uchio et al. (1981).

uli were represented as a percent of the mean of the two nearest responses to the standard (Dickens, 1978, 1981). The size of the EAG depolarization was considered to be a measure of the relative number of responding acceptors (Payne, 1975; Dickens and Payne, 1977). Responses were compared for significant differences using a $t$ test for two means (Ostle, 1963).

## RESULTS AND DISCUSSIONS

The mean responses of *T. bacharides* females to the 1-hexanol standard (100 $\mu$g) was $-0.72$ mV (SE = 0.13; $N = 5$).

*Responses to Alcohol and Aldehydes.* EAGs elicited by primary alcohols and aldehydes of varying chain lengths were maximal for the 5- and 6-carbon alcohols, and the 7-carbon aldehyde (Figures 1 and 2). Although EAGs to the saturated 6-carbon alcohol were greater than those elicited by the corresponding aldehyde, response to the isomer *trans*-2-hexenal was significantly greater than responses to any other 6-carbon alcohol or aldehyde tested ($P < 0.10$) (Figure 3).

Visser and his colleagues (see Visser, 1983) hypothesized that the orien-

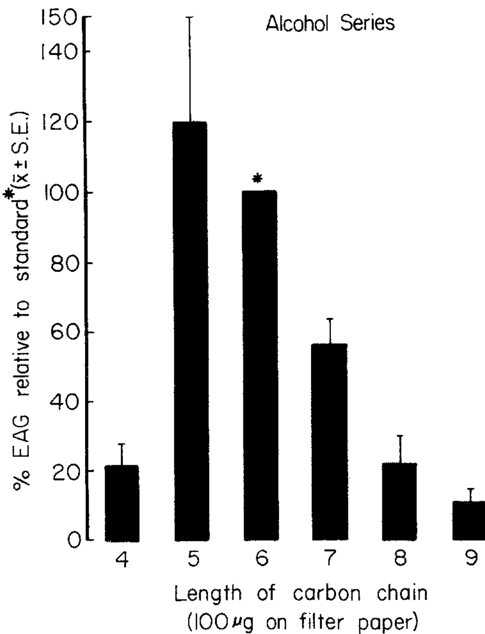



FIG. 1. Mean EAGs of female *T. bacharides* to 100-$\mu$g dosage of saturated primary alcohols of varying chain lengths.

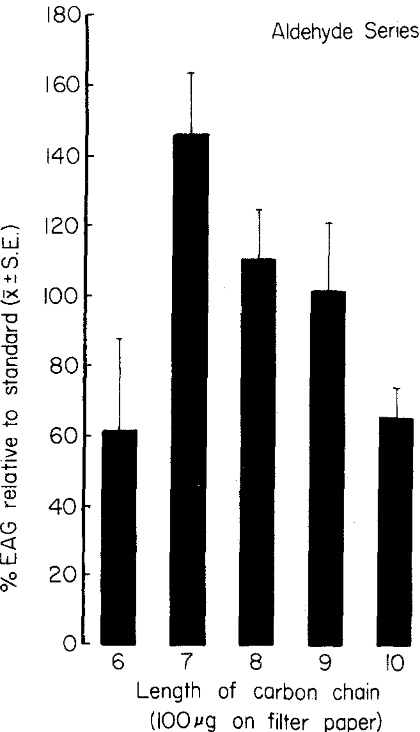



FIG. 2. Mean EAGs of female *T. bacharides* to 100-$\mu$g dosage of saturated aldehydes of varying chain lengths.

tation of phytophagous insects to their hosts involved saturated and monounsaturated 6-carbon aldehydes and alcohols derived from oxidative degradation of plant lipids. The fact that *T. bacharides* females have receptors responsive to both 6-carbon alcohols and aldehydes, and especially *trans*-2-hexenal, a compound present in closely related Compositae (MacLeod et al., 1982), could be indicative of the role of these odors in host location and selection of oviposition sites.

Greater responsiveness to the saturated leaf alcohol relative to the corresponding aldehyde was reported for the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Visser, 1979) and the boll weevil, *Anthonomus grandis* Boh. (Dickens, 1984). *trans*-2-Hexenal was the most active monounsaturated 6-carbon aldehyde for both the carrot rust fly, *Psila rosae* (F.) (Guerin and Visser, 1980) and the cereal aphid, *Sitobion avenae* (F.) (Yan and Visser, 1982). Peak responsiveness of antennal receptors to the 7-carbon aldehyde in a series of aldehydes of varying chain lengths was found for several lepidopterous species (Van der Pers, 1981), several dipterous species (Guerin and Städler, 1982) and *A. grandis* (Dickens, 1984).

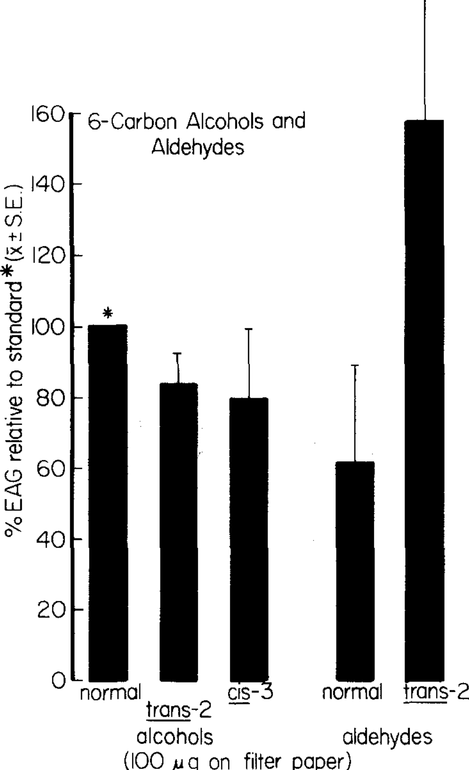
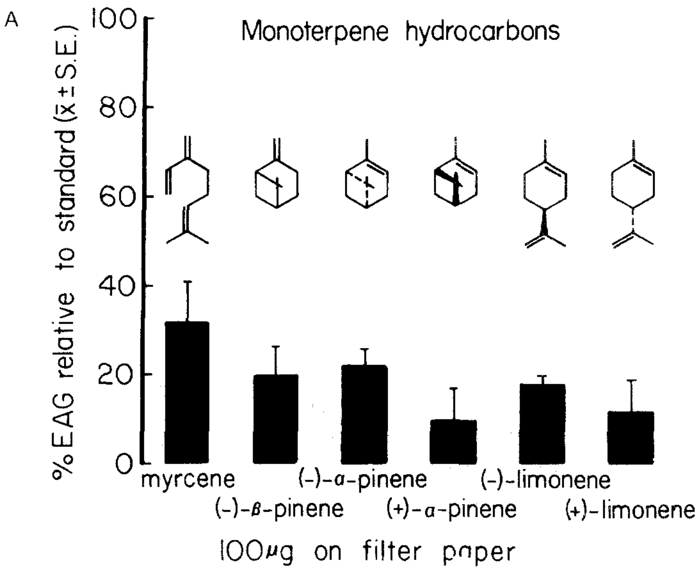



FIG. 3. Mean EAGs of female *T. bacharides* to 100-μg dosage of 6-carbon saturated and monounsaturated primary alcohols and aldehydes.

*Response to Monoterpenes.* In general, *T. bacharides* females had more antennal acceptors reponsive to oxygenated monoterpenes than to monoterpene hydrocarbons (Figure 4). Among the monoterpene hydrocarbons tested, myrcene elicited the greatest response, while the (−)-enantiomers of α-pinene and limonene were more active than their optical antipodes; none of these differences were significant. Oxygenated monoterpenes elicit greater EAGs than monoterpene hydrocarbons in several other oligophagous insects including *L. decemlineata* (Visser, 1979), the oak flea weevil, *Rhychaenus quercus* L. (Kozlowski and Visser, 1981), *P. rosae* (Guerin and Visser, 1980), and *A. grandis* (Dickens, 1984).

*Response to Sesquiterpenes.* Of the three sesquiterpenes tested, both β-caryophyllene and β-bisabolol elicited significant EAGs (Figure 5A). β-Caryophyllene is a major component of the volatile bouquet of several Compositae including globe artichoke, *Cyanara scolymus* L. (Buttery et al., 1978) and *Artemesia capillaris* Thunb. (Harada and Iwasaki, 1982), and is found in the essential oils of three *Chrysanthemum* species (Uchio et al., 1981).



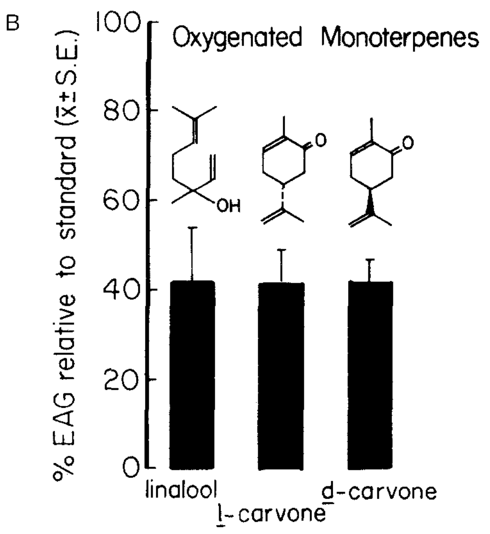
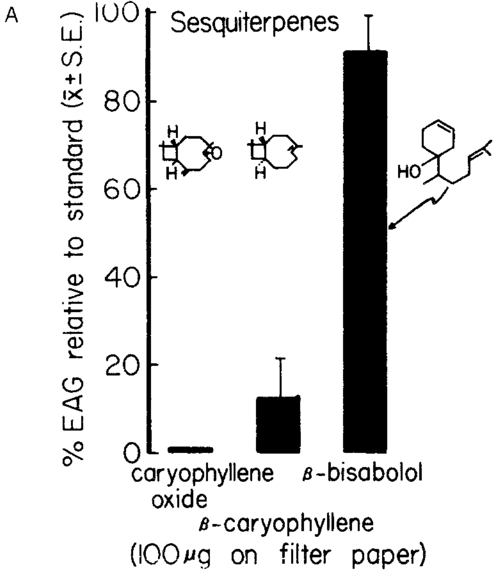




FIG. 4. Mean EAGs of female *T bacharides* to 100-μg dosage of monoterpene hydrocarbons (A) and oxygenated monoterpenes (B).



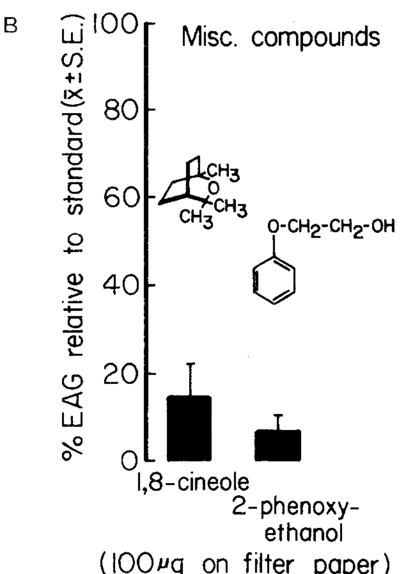




FIG. 5. Mean EAGs of female *T bacharides* to 100-μg dosage of several sesquiterpenes (A) and other compounds (B).

Table 2. Antennal Olfactory Responsiveness of *T. bacharides* Female to Volatiles Emanating from Crushed Leaves[a] of Several Plant Species

| Species | Common name | % EAG relative to standard |
|---|---|---|
| Compositae | | |
| Astereae | | |
| *Baccharis halimifolia* L.[b] | Sea-myrtle | 103.4 |
| *B. neglecta* Britt.[b] | New deal weed | 103.2 |
| *B. salicina* T.&G.[b] | Willow baccharis | 111.3 |
| *B. bigelovii* Gray. | Bigelow baccharis | 102.9 |
| *B. brachyhylla* Gray. | | 92.0 |
| *B. glutinosa* (R.&P.) Pers. | Seepwillow baccharis | 82.8 |
| *B. pilularis* D.C. | Coyote bush | 122.6 |
| *B. pteronioides* D.C. | Yerba de pasmo | 67.6 |
| *Aster novae-angliae* L. | New England aster | 72.5 |
| *Erigeron aurantiacus* Regel. | Double orange daisy | 49.7 |
| *Ericameria austrotexana* M.C. Johnst. | False broomweed | 122.0 |
| *Gutierrezia sarothrae* (Pursh.) Britt. & Rusby. | Broom snakeweed | 122.0 |
| *Isocoma drummondii* (T.&G.) Greene. | Drummond goldenweed | 150.5 |
| Anthemideae | | |
| *Artemisia tridentata* Nutt. | Big sagebrush | 130.4 |
| *Chrysanthemum morifolium* Ramat. | Florist's mum | 135.8 |
| Eupatorieae | | |
| *Eupatorium compositifolium* Walt. | Yankee weed | 95.4 |
| Heliantheae | | |
| *Helianthus annus* L. | Common sunflower | 38.8 |
| Inuleae | | |
| *Antennaria fallax* Greene. | Largeleaf pussy's toes | 57.5 |
| Cichorieae | | |
| *Taraxacum officinale* Wiggers. | Common dandelion | 42.1 |
| Fagaceae | | |
| *Quercus* sp. | Oak | 8.0 |
| Magnoliaceae | | |
| *Magnolia* sp. | Magnolia | 84.4 |
| Rosaceae | | |
| *Rosa* sp. | Rose | 57.0 |
| Leguminosae | | |
| Caesalpinioideae | | |
| *Cercis canadensis* L. | Red bud | 32.3 |
| Papilionideae | | |
| *Trifolium repens* L. | White clover | 107.6 |
| Malvaceae | | |
| *Gossypium hirsutum* L. | Cotton | 32.6 |
| Caprifoliaceae | | |
| *Lonicera* sp. | Honeysuckle | 54.3 |

[a]EAG elicited by 10 × 5-mm leaf section crushed on filter paper.
[b]Known host plants of *Trirhabda bacharides*.

The response elicited by β-bisabolol was greater than the response to any other sesquiterpene ($P < 0.05$) and was equal to or greater than response elicited by any other compound tested with the exception of heptanal and *trans*-2-hexenal (Figures 2, 3, 5A). This fact is interesting since β-bisabolol has only been reported in malvaceous plants (Thomspon et al., 1971). However, sesquiterpenes comprise a large percentage of the volatile components of several other composites (Uchio et al., 1981; Harada and Iwasaki, 1982; MacLeod et al., 1982), and β-bisabolene, a sesquiterpene closely related to β-bisabolol, is the major volatile component of Jerusalem artichoke, *Helianthus tuberosus* L. (MacLeod et al., 1982).

*Response to Other Compounds.* Significant EAGs were recorded to both 2-phenoxyethanol and 1,8-cineole (Figure 5B). 1,8-Cineole is a major constituent of the essential oils of three species of the composite genus *Chrysanthemum* (Uchio et al., 1981).

*Response to Plant Volatiles.* EAGs were elicited by crushed leaves from each of the plant species tested (Table 2). In general, EAGs to volatiles ema-

TABLE 3. PLANTS COLLECTED IN PROPINQUITY TO *T. bacharides* HOST PLANT, *B. neglecta*, NEAR BELTON, TEXAS, APRIL 1983–1984

| Species | Family | Relative abundance | % EAG relative to standard |
|---|---|---|---|
| Forbes | | | |
| *Baccharis neglecta* Britt. | Compositae | Common[a] | 103.2 |
| *Solidago altissima* (L.) | Compositae | Common | |
| *Ratibida columnaris* (Sims) D. Don | Compositae | Very common | |
| *Gutierrezia texana* (DC) T.&G. | Compositae | Scarce | |
| *Helianthus annus* L. | Compositae | Scarce | 38.8 |
| *Erigeron* sp. | Compositae | Scarce | 49.7 |
| *Taraxacum officinale* Wiggers | Compositae | Scarce | 42.1 |
| *Daucus carota* L. | Umbelliferae | Common | |
| *Verbena* sp. | Verbenaceae | Common | |
| Shrubs or trees | | | |
| *Quercus* sp. | Fagaceae | Scarce | 8.0 |
| *Cercis canadensis* L. | Caesalpinioideae | Scarce | 32.3 |
| *Juniperus ashei* Buchh. | Cupressaceae | Common | |
| Grasses | | | |
| *Panicum oligosanthus* Schult. | Gramineae | Common | |
| *Bothriochloa* sp. | Gramineae | Common | |
| *Buchloe dactyloides* (Nutt.) Engelm. | Gramineae | Common | |
| *Sorghum halepense* (L.) | Gramineae | Common | |
| *Bramus tectorum* L. | Gramineae | Very common | |

[a]Known host plant of *T. bacharides*.

nating from *Baccharis* and other composit species were greater than those elicited by species from other families. One notable exception was white clover, which elicited a response equal to or greater than several composites. However, when one considers only plants occurring in propinquity to *T. bacharides* host, *B. neglecta*, EAGs elicited by host volatiles were at least two times greater than those elicited by other coinhabiting plants (Table 3).

The response of antennal receptors of *T. bacharides* to volatiles emanating from each of the plant species tested might be explained by the stimulating effect of general green leaf volatiles in each (Visser, 1979, 1983; Kozlowski and Visser, 1981). However, this assumption is not meant to exclude effects of other more specific volatiles present in the host plant, as indicated by greater response of *T. bacharides* to its host relative to other coinhabiting species. Therefore, it appears likely that input from a medley of volatiles as well as other sensory modalities perceived by the insect in its environment may be important in the location of suitable hosts (Visser and Avé, 1978; Visser, 1983; Dickens, 1984).

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# KAIROMONES FOR THE EGG PARASITE *Trichogramma evanescens* WESTWOOD[1] I. Effect of Volatile Substances Released by Two of Its Hosts, *Pieris brassicae* L.[2] and *Mamestra brassicae*, L.[3]

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**Abstract**—In a four-armed airflow olfactometer *Trichogramma evanescens* Westwood females were attracted by a volatile substance(s) released by virgin females of the great cabbage white butterfly, *Pieris brassicae* L. Males or recently mated females did not cause attraction. Furthermore, *T. evanescens* was also attracted by volatiles released by calling virgin cabbage moths, *Mamestra brassicae* L. However, the parasites did not respond to (*Z*)-11-hexadecenylacetate (the main component of the sex pheromone of *M. brassicae*), a crude hexane extract of the sex pheromone gland, or to males or recently mated females.

**Key Words**—*Trichogramma evanescens, Hymenoptera, Trichogrammatidae, Pieris brassicae,* Lepidoptera, Pieridae, *Mamestra brassicae, Noctuidae,* kairomone, sex pheromone, (*Z*)-11-hexadecenylacetate.

## INTRODUCTION

Many parasitic Hymenoptera are known to be guided by chemical stimuli in the process of host-habitat and host location (Vinson, 1981; Weseloh, 1981). From a distance, a parasite may be attracted by volatile chemicals emanating from the host habitat, the host, or both. The latter substances are called kairomones (for definition see Brown et al., 1970; Nordlund and Lewis, 1976). Once it has ar-

[1] Hymenoptera: Trichogrammatidae.
[2] Lepidoptera: Pieridae.
[3] Lepidoptera: Noctuidae.



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rived in the host habitat, the parasite may be arrested by contact kairomones. This paper deals with some of the volatile kairomones involved in the searching behavior of *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae). Contact kairomones will be discussed in a following paper (Noldus and van Lenteren, 1985).

Some volatile stimuli involved in the searching process of *Trichogramma* spp. have already been demonstrated. For example, plant odors may influence the rate of parasitization by *Trichogramma* spp. in the field (Bar et al., 1979; Altieri et al., 1981). Recently, volatiles released by the host have been shown to function as a kairomone for the parasite: Lewis et al. (1982) observed increased rates of parasitism by wild *Trichogramma* spp. elicited by volatiles present in the abdominal tip and those in a pinkish excretion (supposed to be meconium) as well as by a synthetic sex pheromone blend of *Heliothis zea* (Boddie). The same phenomenon has been found concerning the egg parasite *Telenomus remus* Nixon, in reaction to synthetic sex pheromone components and to an extract from abdominal tips of its host *Spodoptera frugiperda* (Smith) (Nordlund and Lewis, 1983).

During the past few years efforts have been made in the Netherlands to develop biological control of five lepidopteran species in cabbage crops by means of inundative releases of *T. evanescens* (van Lenteren et al., 1982; van der Schaaf et al., 1984). The results described above led us to examine whether or not volatiles released by the adults of hosts of *T. evanescens,* and encountered by this parasite in Dutch cabbage fields, can serve as a kairomone and whether these substances might be used to manipulate parasite behavior. The study was conducted with two host species: the great cabbage white butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae), and the cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae).

We examined the response of *T. evanescens* to virgin females, males, and mated females of *P. brassicae.* In the case of *M. brassicae,* we attempted to determine the reaction of the parasite to the sex pheromone. Therefore, experiments were carried out with the main component (*Z*)-11-hexadecenylacetate, with a crude extract of the sex pheromone gland, and with calling virgin moths. Additionally, responses to males and mated females of this species were tested.

## MATERIALS

The *T. evanescens* strain used for the experiments was collected in the Netherlands in 1981 from eggs of *M. brassicae* in cabbage. It has been reared in the laboratory since then on eggs of *Ephestia kuehniella* Zeller. Adult wasps were maintained on honey in glass tubes at 25°C.

For the experiments, only female wasps with an age varying from 2 to 4 days were used. This age was chosen because wasps younger than 48 hr are apparently not strongly motivated to react to host-seeking stimuli (Smits, 1982).

Previous to an experiment, three eggs of the host species to be tested were offered to each wasp for approximately 1 hr. Only the wasps that were observed to have been parasitizing during the hour of exposure were used in the experiment. Such "experienced" wasps have been shown to commence searching more effectively than inexperienced wasps (Gross et al., 1981).

Pupae of *P. brassicae* and *M. brassicae* were obtained from laboratory rearings of the Departments of Entomology and Animal Physiology, Agricultural University, Wageningen. Some of the pupae were sexed and isolated; after emerging, the adults were kept separate. The adults emerging from the remaining pupae were maintained in Plexiglas cages and were used as a source of eggs. *P. brassicae* was kept at 20°C. *M. brassicae* was kept under a reversed photoregime of 18:6 light–dark (photophase: 20°C; scotophase: 15°C), in order to make experiments possible during the day.

Synthetic (*Z*)-11-hexadecenylacetate was obtained from Dr. K.V. Terytze, Humboldt-Universität zu Berlin, Berlin-Malchow, DDR. It was formulated on rubber septa (pieces of tubing) with a dosage of 2.0 mg/septum. Septa of this kind have been used in field traps for monitoring purposes for some years (Terytze and Adam, 1981). The crude extract of the sex pheromone gland of *M. brassicae* came from Prof. Dr. H.J. Bestmann, Universität Erlangen-Nürnberg, Erlangen, BRD. It was dissolved in *n*-hexane to a concentration of 10 female equivalents (FE) per milliliter. The rubber septa as well as the gland extract solution were stored at −25°C.

## METHODS

The experiments were carried out in a slightly altered version of the four-armed airflow olfactometer described in detail by Vet et al. (1983). Because their olfactometer was too large for *T. evanescens*—the wasps spent too much time walking from one flow field to another and were hardly visible on the video monitor—some modifications had to be introduced in order to make observations of this parasite possible. The size of the exposure chamber was halved (ray of crescents: 67.5 mm; inner height: 5 mm; inner diameter of stainless-steel tubes: 3 mm), the bottom was made of white acrylate, and for the construction of the upper part (odorless) silicone glue was used. In order to create flow fields with sharp boundaries, the flow rate was reduced to 27 ml/min through each arm.

For the experiments with *P. brassicae* the olfactometer was supplied with a large glass spherical container (volume 2 liters) in which the butterflies could move freely. This container could easily be connected to the apparatus without the flow rate being altered. One to three butterflies were put into the container, and the control arms of the olfactometer remained empty. In order to prevent fatigue, the butterflies were replaced after every 10 replications.

In the first experiment concerning *M. brassicae,* we tested synthetic (*Z*)-

11-hexadecenylacetate, by offering one rubber septum [with 2.0 mg (*Z*)-11-IHD A on it] in one arm of the olfactometer; the others remained empty. The septum was replaced after every 10 observations.

For the experiments with the crude extract of the sex pheromone gland, four pieces of Whatman No. 1 filter paper (4 $cm^2$) were used, one of which was treated with 0.1 ml of the hexane extract (10 FE/ml) so that 1 FE was present on the paper. The three others were treated with 0.1 ml *n*-hexane only. A second series was conducted with a load of 10 FE by using 1 ml of the solution on the test filter paper and 1 ml of *n*-hexane on the remaining three filter papers. The experiments were started as soon as the solvent had evaporated. The filter papers were also replaced after every 10 observations.

The experiments with living moths of *M. brassicae* required more preparation. In order to obtain an airstream through the olfactometer containing sex pheromone molecules released by calling virgin females, some conditions had to be met. First of all, the experiments had to be conducted during the period of calling activity of moths in the field. This is known to be highest between 1.5 and 1 hr before dawn (Van de Veere, personal communication). With the photo regime used, we could thus determine at what time during the day the tests had to be done. Secondly, for calling activity of *M. brassicae*, a dark environment is necessary. For this reason some special adaptations were introduced to make experiments with moths in the brightly illuminated olfactometer set-up possible (Figure 1). In a light-tight compartment outside the set-up, a Plexiglas cylinder (11.5 × 4.5 cm) was situated, connected by (odorless) silicone tubes to the first and third vial of one of the olfactometer arms. A virgin moth was put into the cylinder; with the aid of a light-lock and a very weak red darkroom

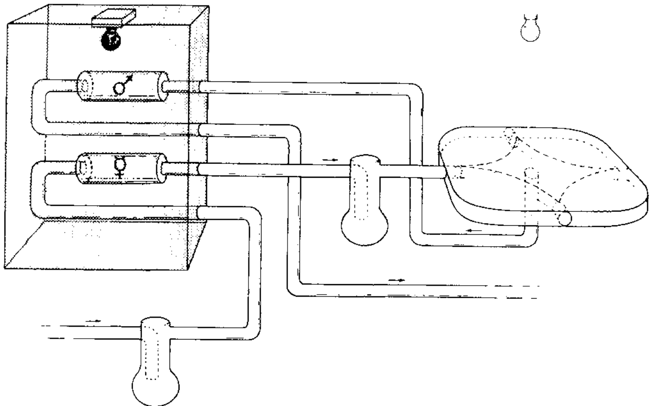

FIG. 1. Schematic representation of the adaptations of the four-armed airflow olfactometer of Vet et al. (1983) to experiments with male and with calling female moths of *Mamestra brassicae*.

lamp, the moth could be observed without being disturbed by the fluorescent light. Thus we could determine whether the calling position (protrusion of the abdominal tip) was assumed. But, as the calling position is no guarantee that sex pheromone release is actually taking place (Pope et al., 1982), another verification was necessary. For this purpose we used the typical response of the male moth, consisting of wing fanning, walking, and flying (Novak et al., 1979), as proof of the presence of sex pheromone molecules in the air stream. A second cylinder, containing a male moth that had not yet mated, was placed in the dark compartment. This cylinder was connected between the air outlet of the olfactometer and the vacuum compressor. The experiment was not started until both the female calling position and the male wing fanning response had been observed. The moths were replaced after every 10 replications.

The experiments were conducted in a climate room at a temperature of 25°C and a relative humidity of 60 ± 5%. Experiments consisted of series of at least 40 observations. They were carried out according to the procedure of Vet et al. (1983), except for the "number of first choices." This measurement was abandoned because, during preliminary observations, the wasps turned out to cross the "first-choice line" before the odor fields could have been formed. After an insect had entered one of the arms of the olfactometer and had stayed there for more than 2 min, it was said to have made a "final choice." Statistical analysis of the results was also done according to Vet et al. (1983).

## RESULTS

*Pieris brassicae.* The first experiment was performed with virgin females of *P. brassicae.* These were often observed in the rearing cage sitting in a typical position, in which the wings were spread out, the abdomen was raised slightly, and the lateral lobes of the genital apparatus were more or less turned aside. This posture stands somewhat halfway between the "mate-refusal posture" and the "mate-acceptance posture" which normally occur only during the mating sequence, preceded by male stimulation (Chovet, 1982). It was never adopted by males or recently mated females. Thinking of the calling position known of many moth species, we wondered whether a volatile substance was released at that moment, and what the effect on the parasite might be. The results are given in Table 1 A. Attraction of the parasite is distinctly shown by the fact that all the final choices were made for the *P. brassicae* field and that the average percentage of time spent in this field is significantly longer than the time spent in the other odor fields.

In order to determine whether the attraction was caused by an odor specifically released by the virgin females, and not by an odor present in all adult butterflies of *P. brassicae,* control experiments were carried out with males and

females that had been mated the day before the experiment. It is very unlikely that such females release odors that are related to the mating behavior, because *P. brassicae* females do not mate again until five days or more after the first copulation (David and Gardiner, 1961). The results of these experiments are given in Table 1B and C. No preference is shown in this case: the numbers of final choices as well as the time allocation are randomly distributed over the four flow fields.

From these results we may conclude that virgin females of *P. brassicae* can release a volatile substance which acts as a kairomone for *T. evanescens* and which is not released by males or recently mated females.

*Mamestra brassicae. M. brassicae* is a noctuid and thus represents a group on which several sex pheromone studies have been done. Szentesi et al. (1975) demonstrated the presence of a sex pheromone in *M. brassicae*. Subsequently several examiners have tried to find out its chemical composition. The main

TABLE 1. RESPONSE OF FEMALE *Trichogramma evanescens* TO ODOR OF LIVING BUTTERFLIES OF *Pieris brassicae IN AN OLFACTOMETER*[a]

| Bufferflies tested | Response | $N$ | Odor field 1 | 2 | 3 | 4 | $P$ |
|---|---|---|---|---|---|---|---|
| A. Virgin females | No. final choices | 17 | 17 | | | | $3.6 \times 10^{-12}$ |
| | Mean % time spent/field | 40 | 67.9 | 10.7 | 15.3 | 6.1 | |
| | Friedman rank sum | | 135.5 | 82 | 94.5 | 88 | $5.8 \times 10^{-7}$ |
| B. Males | No. final choices | 12 | 2 | 5 | 2 | 3 | 0.84 |
| | Mean % time spent/field | 50 | 17.1 | 32.5 | 27.7 | 22.7 | |
| | Friedman rank sum | | 112.5 | 142.5 | 116.5 | 128.5 | 0.076 |
| C. Mated females | No. final choices | 14 | 4 | 4 | 4 | 2 | 0.48 |
| | Mean % time spent/field | 50 | 26.1 | 27.4 | 24.8 | 21.7 | |
| | Friedman rank sum | | 128.5 | 128 | 119.5 | 124 | 0.88 |

[a] 1 = *P. brassicae*, 2 + 3 + 4 = air.

component has turned out to be (*Z*)-11-hexadecenylacetate, but considerable disagreement exists about the presence and ratios of minor components (Bestmann et al., 1978; Farine et al., 1981; Novak et al., 1979; Struble et al., 1980). We have examined the response of *T. evanescens* to synthetic (*Z*)-11-hexadecenylacetate, to a crude extract of the sex pheromone gland, and to the volatiles released by calling virgin females of *M. brassicae*.

In the first experiment, in which (*Z*)-11-HDA was offered, no attraction of *T. evanescens* could be measured. The data in Table 2A show that the numbers of final choices as well as the average percentage of time spent in the different flow fields do not differ significantly. The same applies to both loads of the crude hexane extract of the sex pheromone gland (Table 2B and C).

Table 2D however shows that as soon as the volatiles of *M. brassicae*, released by calling virgin moths, are offered, significant attraction of *T. evanescens* occurs. Both at the final-choice level and as far as the time allocation per odor field are concerned, the parasites demonstrate a distinct preference for the *M. brassicae* field.

That the attraction is indeed caused by the sex pheromone and not by some other volatile is strongly suggested by the fact that *M. brassicae* responded in a typical way to the same volatiles and by the control experiments in which males or recently mated females were offered. Table 2E and F shows that *T. evanescens* was neither attracted by males nor by females mated the day before the experiment.

The data in Table 2 suggest that the sex pheromone of *M. brassicae*, in the form released by calling virgin moths, can act as a volatile kairomone for *T. evanescens*.

## DISCUSSION

The first three experiments have shown that virgin females of *Pieris brassicae* can release a volatile kairomone for *T. evanescens*. With the results of *Heliothis zea* (Lewis et al., 1982), *Spodoptera frugiperda* (Nordlund and Lewis, 1983), and *Mamestra brassicae* (this paper) in mind, one might expect that *P. brassicae* produces a sex pheromone. However, the mating behavior of *P. brassicae* has been studied in detail by several authors, and none of them mentions the existence of a female sex pheromone (David and Gardiner, 1961; Chovet, 1982; Feltwell, 1982). In general, mate location in butterflies is assumed to occur mainly on the basis of visual stimuli (Myers, 1972; Scott, 1974). The only chemical stimuli that are known to play a role in the mating sequence of *P. brassicae* are short-range volatiles originating from the androconial scales of the male (Bergström and Lundgren, 1973). In the meantime, we have, without success, tried to examine whether the volatile substance released by the virgin females has an intraspecific function too.

Table 2. Response of Female *Trichogramma evanescens* to Odor of Various Substances Originating from *Mamestra brassicae* in an Olfactometer[a]

| Material tested | Response | N | Odor field | | | | P |
|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | |
| A. Synthetic (*Z*)-11-hexadecenylacetate | No. final choices | 8 | 4 | 1 | 2 | 1 | 0.11 |
| | Mean % time spent/field | 40 | 28.7 | 21.7 | 27.2 | 22.4 | |
| | Friedman rank sum | | 110 | 91 | 102 | 97 | 0.40 |
| B. Crude hexane extract of the sex-pheromone gland (1 FE) | No. final choices | 4 | 3 | 1 | | | 0.051 |
| | Mean % time spent/field | 40 | 28.1 | 23.6 | 28.4 | 19.9 | |
| | Friedman rank sum | | 102 | 93 | 110 | 95 | 0.42 |
| C. Crude hexane extract of the sex-pheromone gland (10 FE) | No. final choices | 8 | 3 | 1 | 3 | 1 | 0.32 |
| | Mean % time spent/field | 40 | 24.0 | 28.8 | 23.2 | 24.0 | |
| | Friedman rank sum | | 103 | 104 | 93 | 100 | 0.76 |
| D. Sex pheromone released by living calling female moth | No. final choices | 9 | 7 | 1 | 1 | | 0.0013 |
| | Mean % time spent/field | 44 | 39.4 | 23.6 | 21.9 | 15.1 | |
| | Friedman rank sum | | 140.5 | 116 | 96.5 | 87 | $3.8 \times 10^{-5}$ |
| E. Male moth | No. final choices | 1 | 1 | | | | |
| | Mean % time spent/field | 40 | 26.8 | 20.9 | 23.3 | 29.0 | |
| | Friedman rank sum | | 103 | 87 | 94 | 116 | 0.068 |
| F. Mated female moth | No. final choices | 2 | | 1 | 1 | | |
| | Mean % time spent/field | 40 | 16.9 | 31.1 | 34.1 | 17.9 | |
| | Friedman rank sum | | 89.5 | 113 | 108 | 89.5 | 0.075 |

[a] 1 = material tested, 2 + 3 + 4 = air.

The results of the experiments with *M. brassicae* showed that *T. evanescens* is attracted by volatiles which are probably the sex pheromone released by calling virgin females, but not by the main component (*Z*)-11-hexadecenylacetate, nor by a crude extract of the sex-pheromone gland (at the concentrations tested). This suggests that there are differences between the pheromone released into the air by the insect and the contents of the pheromone gland. This phenomenon has been demonstrated in other species too; Tumlinson et al. (1982) list some possible causes for its occurrence. Our results affirm their statement that bioassays with sex pheromones should be based on materials collected from living calling females, considering that it is the only way to be sure one is testing the proper material.

Apparently some component(s) other than only (*Z*)-11-HDA is (are) necessary for the attraction of *T. evanescens* in the olfactometer, for the latter did not elicit a response of the parasite. In the future, blends of components should be tested separately to determine the exact cause of the attraction. This could be done after analysis of a filter collection of the pheromone, such as might be done with a Porapak device (Byrne et al., 1975; Tumlinson et al., 1982).

Finally some remarks may be made on the possible function of the kairomones in the searching behavior of the parasite. Volatiles released by virgin females do not necessarily have a direct temporal or spatial correlation with the moment or site of oviposition. Therefore, we do not expect the egg parasite to be guided directly towards the place of release. The kairomone might rather lead the parasite to an area where mating is in progress and where oviposition is thus likely to take place or to have taken place. At the moment a single female host is calling, eggs of conspecifics may already be present in the surroundings. Hence, responding to the sex pheromone of the host seems adaptively favorable for an egg parasite. This also applies to the sex pheromone of *M. brassicae,* which is released at night. It may yet be detectable for *T. evanescens,* which is active during the day, as pheromone molecules are known to be absorbed by the surroundings of the calling moths (e.g., plants) and to evaporate very slowly (Farkas and Shorey, 1974; Lewis, personal communication). Furthermore, responding to the sex pheromone of the host might also serve to avoid competition with predators. Thus, it may be important for an egg parasite to find the host eggs shortly after oviposition and parasitize them as soon as possible, as some predators are known to dislike eggs that have turned black as the result of a developing parasite larva (Lewis et al., 1982).

Actual finding of the host eggs may occur only after an intense search stimulated by contact kairomones left by ovipositing hosts. These substances will be discussed in a following paper (Noldus and van Lenteren, 1985).

We believe that the volatile effects described above may promise exciting possibilities for the use of sex pheromones in insect pest control, as has recently been stressed by Greenblatt and Lewis (1983): disruption of mating of the pest may be attended by enhancement of parasitization by the parasite.

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# KAIROMONES FOR THE EGG PARASITE *Trichogramma evanescens* WESTWOOD[1]
## II. Effect of Contact Chemicals Produced by Two of Its Hosts, *Pieris brassicae* L.[2] and *Pieris rapae* L.[2]

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**Abstract**—In observation-cage experiments some new contact kairomones for the egg parasite *Trichogramma evanescens* Westwood are demonstrated. *T. evanescens* females search significantly longer on cabbage leaves treated with the wing scales of two hosts, *Pieris brassicae* L. and *P. rapae* L. Further, egg washes of *P. brassicae* containing an oviposition deterrent pheromone for the butterflies, were found to have a contact-kairomonal effect on the parasite. *T. evanescens* females search significantly longer on cabbage leaves sprayed with a methanol or water wash of *P. brassicae* eggs than on leaves treated with the solvent only.

**Key Words**—*Trichogramma evanescens,* Hymenoptera, Trichogrammatidae, *Pieris brassicae*, *Pieris rapae*, Lepidoptera, Pieridae, kairomone, oviposition deterring pheromone, accessory gland, egg parasite, tricosane.

## INTRODUCTION

In a previous paper we discussed the significance of volatile kairomones for the egg parasite *Trichogramma evanescens* Westwood in host-habitat location (Noldus and van Lenteren, 1985). Now we present the results of experiments concerning the involvement of contact kairomones in host location by this parasite.

The first demonstration of contact-kairomonal effects for *Trichogramma* spp. occurred some 50 years ago. At that time, Laing (1937) found that female *T.*

[1] Hymenoptera: Trichogrammatidae.
[2] Lepidoptera: Pieridae.



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*evanescens* reacted to an "odor trail" left at oviposition sites by adult *Sitotroga cerealella* (Olivier) moths. Contact with the host trail led to strong klinokinetic and klinotactic responses, the parasite often returning to the same place. Later, Lewis et al. (1971) showed a comparable effect with *Heliothis zea* (Boddie). They found that the wing scales were the source of the kairomone (Lewis et al., 1972) and that the main component was tricosane (Jones et al., 1973). *T. evanescens*, *T. pretiosum* Riley, and *T. achaeae* Nagaraja and Nagarkatti also responded to the kairomone with an intensified searching behavior (Lewis et al., 1975). Application of this substance in the field can lead to a considerable increase in the rate of parasitism of *H. zea* eggs, provided that a proper treatment pattern is used (Lewis et al., 1979; Gross, 1981). Recently, the same effect has been shown with the wing scales of *Mamestra brassicae* L., one of the main lepidopteran pests in cabbage fields in the Netherlands (Smits, 1982). Two other hosts of *T. evanescens* in cabbage are *Pieris brassicae* L. and *P. rapae* L. The effect of the wing scales of these species on the searching behavior of the parasite has not been investigated before and was the subject of the present study.

Besides wing scales, another possible source of kairomone has been tested. Rothschild and Schoonhoven (1977) have found that *P. brassicae* females deposit an oviposition-deterring pheromone on and around the eggs during oviposition. This pheromone probably originates from the accessory gland (Behan and Schoonhoven, 1978). It can be collected by washing eggs with methanol or water. *P. brassicae* lays significantly fewer eggs on leaves that have been treated with such an extract than on untreated leaves. The material acts mainly at contact and in a limited degree at a short distance (Klijnstra, 1982). We wondered whether this cue, informing the host about the presence of eggs of conspecifics, might also be used by the parasite; however, in the latter case, resulting not in emigration but in arrestment. Therefore, we examined the influence of a methanol and a water wash of *P. brassicae* eggs on the searching behavior of the parasite.

## MATERIALS

The *T. evanescens* strain used for the experiments was the same as mentioned in the previous paper (Noldus and van Lenteren, 1985). It was collected in the Netherlands in 1981 from eggs of *M. brassicae* in cabbage, and has been reared in the laboratory since then on eggs of *Ephestia kuehniella* Zeller. Adult wasps were maintained on honey in glass tubes at 25°C. Only experienced female wasps with an age varying from 2 to 4 days were used (see Noldus and van Lenteren, 1985).

Pupae of *P. brassicae* and *P. rapae* were obtained from a laboratory rearing of the Department of Animal Physiology, Agricultural University, Wageningen. The adults were kept in Plexiglas cages at 20°C.

The cabbage plants, necessary for the experiments and for collecting eggs, originated from R. Zwaan B.V., De Lier, and from Duphar B.V., 's Graveland.

The egg washes of *P. brassicae* were obtained from J.W. Klijnstra, Department of Animal Physiology, Agricultural University, Wageningen. The methanol solution had a concentration of 500 EE/ml (EE = egg equivalent, see Klijnstra, 1982). The concentration of the water solution was 250 EE/ml.

## METHODS

In order to determine whether a substance had a contact-kairomonal effect on *T. evanescens*, the searching time spent on a cabbage leaf treated with the substance to be tested was compared to the searching time spent on an untreated leaf. A Plexiglas observation cage with a green rear side and an open front side was used (Figure 1). In the cage two glass tubes with cabbage leaves (about 25 $cm^2$) were placed so that the leaves were hanging above a third tube from which wasps could be released. Stimulated by the light of a fluorescent tube, hanging above the set-up, the wasps flew up and could land on a leaf. From that moment onwards the wasps were observed until they left the leaf by flying up or walking away along the petiole, and the time spent searching was recorded. The observations were eased by a mirror placed behind the three tubes. To compensate for a possible effect of asymmetrical influences from outside the set-up, the position of the leaves was alternated at the midpoint of a series of observations.

The experiments were carried out in a climate room at a temperature of 20°C. Possible differences in average searching time were tested for significance by means of a Mann-Whitney U test ($\alpha$ = 0.05).

For the treatment of a cabbage leaf with host scales, a clean paintbrush

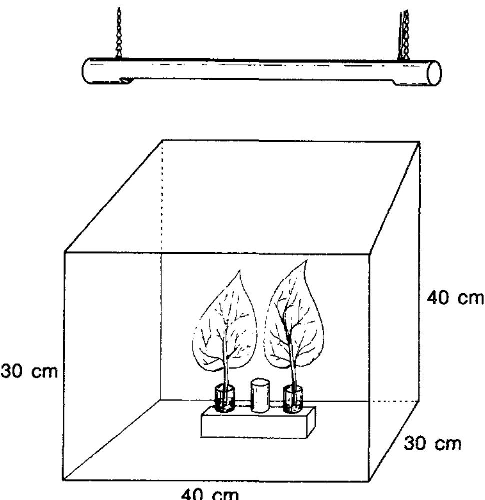


FIG. 1. Plexiglas observation cage. Wasps are released from the vial placed between the two leaves.

was gently moved over the fore and hind wings of an adult female butterfly. The scales collected in this way were spread out over the upper and lower surface of a clean cabbage leaf. This was repeated until both sides were uniformly covered with scales. The control leaf was not treated.

In the case of the egg wash of *P. brassicae*, a cabbage leaf was covered with a very thin layer of the extract by means of a Desaga® chromatographic sprayer. This treatment resulted in a large number of tiny droplets on the waxy leaf surface. One millileter of the methanol solution was used; 2 ml of the water solution was necessary for an equal treatment. The control leaves received a similar amount of the solvent only. The experiments were started as soon as the solvent had evaporated.

## RESULTS

*Host Wing Scales*. The results of the experiment on the kairomonal effect of wing scales of the host are shown in Table 1. To obtain an impression of the responsiveness to kairomones of the *T. evanescens* strain used, first a short series of observations with the scales of *Mamestra brassicae* was made ($N = 5$). The kairomonal effect of *M. brassicae* scales on *T. evanescens* has already been demonstrated by Smits (1982), who worked with a strain collected in Moldavia (U.S.S.R.).

For all three species, we note significantly longer searching times on treated leaves. So the wing scales of *M. brassicae*, *P. brassicae*, and *P. rapae* contain a contact kairomone leading to arrestment of the parasite.

During the first part of each experiment (about 40 landings), the number of landings turned out to be equally distributed over both leaves, which indicates that no long-range attraction occurs. Therefore the observations for each experiment were finished as soon as 25 landings had occurred on each leaf.

*Egg wash of P. brassicae.* Table 1 also lists the results of the bioassays with the egg wash of *P. brassicae*. The methanol as well as the water solution, led to a significant arrestment of the parasite compared with the control leaves. So the egg wash of *P. brassicae*, which contains an oviposition-deterring pheromone for the butterflies, also contains a contact kairomone for *T. evanescens*.

In this case also no long-range attraction occurred, and the experiments were finished after 25 landings on each leaf.

## DISCUSSION

This study has revealed the existence of some new kairomone sources for *Trichogramma evanescens*.

First, the fact that wing scales of the host contain a contact kairomone for

TABLE 1. AVERAGE SEARCHING TIME OF FEMALE *Trichogramma evanescens* ON CABBAGE LEAVES TREATED WITH SUBSTANCES ORIGINATING FROM THE HOST AND ON CONTROL LEAVES

| Material tested | Host | Solvent | $N$ | Test leaves[a] | | Control leaves | | $P$ |
|---|---|---|---|---|---|---|---|---|
| | | | | $\bar{t}$ | $r$ | $\bar{t}$ | $r$ | |
| Wing scales | *Mamestra brassicae* | | 5 | 1327 | 634–2143 | 265 | 24–920 | 0.008 |
| | *Pieris brassicae* | | 25 | 543 | 29–1780 | 264 | 10–899 | 0.0128 |
| | *Pieris rapae* | | 25 | 712 | 40–3197 | 221 | 30–448 | 0.00046 |
| Egg wash containing an oviposition-deterring pheromone | *Pieris brassicae* | Methanol | 25 | 177 | 38–480 | 95 | 18–574 | 0.0020 |
| | | Water | 25 | 130 | 60–271 | 83 | 14–171 | 0.0082 |

[a]($\bar{t}$ = average searching time (sec), $r$ = range).
[b]In wing scale experiment control leaves were untreated; in egg wash experiment control leaves were treated with the solvent only.

the parasite, as shown for *Heliothis zea* (Lewis et al., 1972) and *Mamestra brassicae* (Smits, 1982), turns out to apply to *Pieris brassicae* and *P. rapae* too.

The possibility exists that also in this case tricosane, the most active component of the scales of *H. zea* (Jones et al., 1973), is responsible for the kairomonal effect. One might, however, wonder what the function of this substance can be in the field. Saturated long-chain hydrocarbons like tricosane are very common in surface lipids of insects (Nelson, 1978). Arrestment of a parasite after contact with the trail of any other insect is not likely to increase the searching efficiency. It has therefore been suggested that more host-specific components are present in the scales (Lewis, personal communication); kairomonal activity of components other than the four alkanes mentioned by Jones et al. (1973) has, however, not yet been reported.

Smits (1982), who demonstrated the contact-kairomonal effect of the scales of *M. brassicae* for *T. evanescens*, observed a significantly higher number of landings on treated leaves. However, we found an equal number of landings on both leaves. This difference may have been caused by the way the leaves were treated. In our study leaves were treated by moving a scale-covered brush over them, to make sure that only scales were put on the leaf. Smits exposed the plants to living butterflies and let them walk over the leaves, so that other substances may have been present on the surface.

The material collected by washing *P. brassicae* eggs, and which can act as an oviposition deterrent for the butterflies, can also act as a contact kairomone for the parasite. The only other case in which such a double effect is known is *Rhagoletis pomonella* (Walsh) and its larval parasite *Opius lectus* Gahan (Prokopy and Webster, 1978). The pheromonal and the kairomonal effect might be caused by the same compound. However, this can only be verified after the chemical analysis of the *P. brassicae* egg wash has been completed. Another example of the kairomonal effect of host accessory-gland material for an egg parasite has been reported by Strand and Vinson (1982, 1983). They demonstrated that two large proteins, originating from the accessory gland of *Heliothis virescens* F. and present on the chorion of the eggs, serve as an egg-recognition kairomone for *Telenomus heliothidis* Ashmead. They suggest that the proteins might function as an adhesive for the eggs; they do not mention the possibility of a pheromonal use as an oviposition deterrent. Oviposition-deterring pheromones have been demonstrated for two other hosts of *T. evanescens: Trichoplusia ni* (Hübner) (Renwick and Radke, 1980) and *Ostrinia nubilalis* (Hübner) (Dittrick et al., 1983). These, however, originate from larval frass and kairomonal effects are not known.

The adaptive value for an egg parasite of searching longer or more intensively in areas where host eggs have been deposited seems clear. Hence, the accessory-gland material offers interesting possibilities for application in pest control programs. It may have a double effect by deterring the oviposition of *P. brassicae* and enhancing the parasitization activity of *T. evanescens*. However,

before large-scale experiments can be carried out, careful attention will have to be paid to ecological effects of application of such substances. A wrong treatment pattern might result in the same complications as have arisen with the application of moth scale extracts (Lewis et al., 1979). Studies will be necessary to find out which spraying dosage will yield a maximal joint phenomonal-kairomonal effect of the egg wash.

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# ROLE OF OLFACTION IN HOST FINDING BY APHID PARASITOID *Aphidius nigripes* (HYMENOPTERA: APHIDIIDAE)[1]

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**Abstract**—The olfactory responses of the parasitoid *Aphidius nigripes* Ashmead were investigated with a four-field airflow olfactometer. Female and male parasitoids did not respond to potato plants, the food plant of their host. However, females were strongly attracted by odors from a preferred host, *Macrosiphum euphorbiae* (Thomas), and also from less preferred hosts such as *Myzus persicae* (Sulzer) and *Aphis nasturtii* Kalt. Moreover, *A. nigripes* females responded positively to odors from a nonhost aphid, *Rhopalosiphum maidis* Fitch. The honeydew produced by these four aphid species was also very attractive to females. Males did not respond to aphids or honeydew but were highly attracted by odors from conspecific female parasitoids.

**Key Words**—Habitat selection, host selection, kairomones, sex pheromone, potato aphid, *Macrosiphum euphorbiae, Homoptera, Aphididae, Aphidius nigripes*, Hymenoptera, Aphidiidae, olfaction.

## INTRODUCTION

Olfaction is an important mechanism involved in host-habitat location and host location by insect parasitoids (Vinson, 1981; Weseloh, 1981). Olfactometer studies have shown that aphid parasitoids can be attracted by odors produced by the food plant of their host, thus partly explaining the choice of a particular habitat by a searching parasitoid (Read et al., 1970; Schuster and Starks, 1974; Powell and Zhi-Li, 1983). The parasitoids used in these studies also responded to odors

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from their aphid hosts, although in all cases possible contamination of the aphids by traces of plant substances cannot be ruled out.

*Aphidius nigripes* Ashmead is the major primary parasitoid attacking the potato aphid, *Macrosiphum euphorbiae* (Thomas), on potatoes in northeastern North America, and its possible use as a biocontrol agent has been suggested (Shands et al., 1965; Cloutier et al., 1981). In order to evaluate more precisely the potential of *A. nigripes*, laboratory studies on the searching behavior of this parasitoid were initiated (Bauduin, 1982). Bouchard and Cloutier (1984) demonstrated that contact kairomone(s) present in aphid honeydew elicit intense local searching by *A. nigripes*. The present paper deals with olfactory stimuli which may be used in the field by *A. nigripes* parasitoids in their search for hosts. Binary choice tests using a four-field airflow olfactometer were conducted to measure the olfactory responses of *A. nigripes* to plants, aphids, aphid honeydew, and male and female conspecifics.

## METHODS AND MATERIALS

Aphids and parasitoids were obtained and maintained as previously described by Bouchard and Cloutier (1984). Female parasitoids used in experiments were 3–4 days old, mated, and had previously been exposed to aphids except in tests involving odors from conspecifics where test females were 1–2 days old, virgin, and had never had access to aphids. All male parasitoids tested were 1–2 days old, unmated, and had not been exposed to aphids. The species used as test aphids were *M. euphorbiae, Myzus persicae* (Sulzer), *Aphis nasturtii* Kalt., and *Rhopalosiphum maidis* Fitch. The first three species were reared on potato seedlings (cv. Norland), and the corn leaf aphid was reared on barley seedlings (cv. Loyola).

The olfactometer used was virtually identical to that designed by Vet et al. (1983). The apparatus consisted of a four-arm star-shaped test chamber, each arm being connected to a series of two 50-ml glass flasks. The odor source material was placed in the flask closest to the chamber; the second flask contained distilled water to obtain uniform humidity in incoming air. A variable area flowmeter (Cole-Palmer®, max. flow 516 ml/min) was connected to each set of flasks to regulate the incoming airflow through each of the four ports of the chamber. All parts of the apparatus were connected using Tygon® tubing. The airflow was obtained with a vacuum compressor by pumping air out of the chamber through a 1-cm-diam. hole in the center of the floor, thus creating four distinct moving air fields in the chamber. Tests were performed at a flow rate of 300 ml/min through each arm.

Parasitoids to be tested were introduced individually in the chamber through the outflow port on the floor, and they were allowed a maximum of 5 min to make a final choice between the four air fields. A final choice was recorded when the parasitoid exited the chamber through one of the 0.5-cm-diam. ports of the

chamber. Most parasitoids showed a strong anemotactic response and made a final choice within 5 min. The approximate boundaries of the four air fields were drawn on clear acetate placed on the top of the chamber, so before a final choice was made, the time spent by a parasitoid walking in each of the four fields was recorded with a Datamyte 800® event recorder. The presence of an observer near the apparatus did not seem to disturb the insects. Preliminary tests showed that many parasitoids sampled only two or three of the four odor fields before making a final choice. Therefore all experiments were designed as binary choice tests, i.e., parasitoids were given a simple choice between test material odors and control (blank) "odors." As suggested by Vet et al. (1983), we placed test materials in two opposite arms, the two other arms being the control ones. Each experiment was replicated twice with 20 parasitoids tested per replicate. After the first replicate, the whole apparatus was thoroughly washed with hot water and 95% ethanol. For the second replicate, the material tested as the source of odors was placed in the two arms which served as controls for the first replicate, thus counteracting any bias in the system. All experiments were performed at ca. 21°C, and the only source of light in the laboratory was a 60-W incandescent bulb placed 50 cm over the chamber.

The first experiment was conducted with all four arms connected to blank sources to determine if the distribution of parasitoid responses over the four fields of the chamber was random. In one experiment, the following plants were tested as sources of odors: potato seedlings (cv. Norland, 1.5 g fresh foliage and stem); mature, greenhouse-grown potato plant (cv. Norland, 1.5 g fresh foliage); and 4-leaf barley plants (cv. Loyola, 0.80 g fresh foliage). Possible interference with the normal odor of test plants owing to volatiles emanating from the cut edges was minimized by submerging the cut parts in vials filled with water and sealed with Parafilm®. In an experiment involving aphids, 300 individuals at different stages of development were used for each of the two test fields, except when *M. euphorbiae* (the largest of the four species) was the test aphid in which case 150 individuals were used. The honeydew used in another experiment was collected from each of the four aphid species tested as described by Bouchard and Cloutier (1984). Water solutions containing 0.26 mg dry honeydew/$\mu$l were obtained, and a volume of 150 $\mu$l was applied to a piece of absorbant cotton-wool used as the source. Similar cotton-wool pieces treated with distilled water served as control sources. The olfactory response of *A. nigripes* to conspecific males and females was also studied. In this experiment, 30 unmated, 1- to 2-day-old individuals which had never had access to aphids were used as source of odors for each test field.

## RESULTS

The test using blank fields indicated that no detectable bias was present in the system since the number of final choices (11, 9, 9, and 11) and the mean time

spent per field (0.38, 0.38, 0.30, and 0.25 min) did not differ significantly between the four fields ($P > 0.05$). In experiments involving test materials, a positive response in terms of a greater percentage of parasitoids choosing test fields over controls was always confirmed by a significantly longer time spent walking in test fields (Table 1).

*Aphidius nigripes* females did not show any response to either potato seedlings, mature potato leaves, or barley leaves (Table 1, tests 1, 3, 4). Males were tested only with potato seedlings to which they did not respond (Table 1, test 2). Female parasitoids showed significant attraction to all aphid species tested as well as honeydew obtained from them (Table 1, tests 5, 7–10, 12–14). Males did not respond to *M. euphorbiae* or to its honeydew (Table 1, tests 6, 11).

TABLE 1. RESULTS OF TESTING VARIOUS MATERIALS AS ODOR SOURCES TO *A. nigripes* IN A FOUR-FIELD AIRFLOW OLFACTOMETER

| Test | Material tested | Sex tested[a] | % Choosing test fields | Time spent (min, $\bar{X}$ ± SEM) | |
|---|---|---|---|---|---|
| | | | | Tested fields | Control fields |
| Plants | | | | | |
| 1 | Potato seedling | F | 55 | 0.59 ± 0.11 | 0.53 ± 0.10 |
| 2 | Potato seedling | M | 58 | 0.52 ± 0.12 | 0.49 ± 0.09 |
| 3 | Mature potato leaves | F | 48 | 0.23 ± 0.04 | 0.25 ± 0.05 |
| 4 | Barley leaves | F | 52 | 0.17 ± 0.02 | 0.21 ± 0.03 |
| Aphids | | | | | |
| 5 | *M. euphorbiae* | F | 90*[b] | 0.75 ± 0.13 | 0.32 ± 0.06** |
| 6 | *M. euphorbiae* | M | 45 | 0.24 ± 0.04 | 0.24 ± 0.05 |
| 7 | *M. persicae* | F | 85* | 0.31 ± 0.05 | 0.13 ± 0.02** |
| 8 | *A. nasturtii* | F | 85* | 0.30 ± 0.04 | 0.12 ± 0.02** |
| 9 | *R. maidis* | F | 75* | 0.44 ± 0.07 | 0.18 ± 0.04** |
| Honeydew from | | | | | |
| 10 | *M. euphorbiae* | F | 82* | 0.71 ± 0.11 | 0.42 ± 0.09** |
| 11 | *M. euphorbiae* | M | 48 | 0.37 ± 0.07 | 0.36 ± 0.07 |
| 12 | *M. persicae* | F | 78* | 0.36 ± 0.05 | 0.24 ± 0.04** |
| 13 | *A. nasturtii* | F | 88* | 0.34 ± 0.05 | 0.17 ± 0.03** |
| 14 | *R. maidis* | F | 72* | 0.43 ± 0.06 | 0.23 ± 0.04** |
| Conspecifics | | | | | |
| 15 | Females | F | 48 | 0.47 ± 0.06 | 0.40 ± 0.08 |
| 16 | Males | F | 45 | 0.38 ± 0.07 | 0.36 ± 0.04 |
| 17 | Females | M | 88* | 0.67 ± 0.10 | 0.17 ± 0.05** |
| 18 | Males | M | 50 | 0.31 ± 0.04 | 0.30 ± 0.04 |

[a] 40 parasitoids/test.
[b] *$P < 0.05$ ($\chi^2$ test with correction for continuity); **$P < 0.05$ (paired $t$ test).

Females and males were not attracted or repulsed by parasitoids of the same sex (Table 1, tests 15, 18), indicating that within experiments the choices made by parasitoids tested consecutively were probably independent. Females did not respond to males but males were significantly attracted to females (Table 1, tests 16, 17).

## DISCUSSION

The results clearly demonstrate the *A. nigripes* females can use olfactory cues to locate their hosts. Female parasitoids were strongly attracted by odors from aphids and from aphid honeydew. Such attraction should increase the parasitoid's searching efficiency in the field since females would orient towards plants or plant parts on which aphids are present. However, care must be exercised in extrapolating laboratory results to field conditions, as positive responses in an olfactometer do not imply longer-range attraction (Kennedy, 1965).

Other parasitoids have also been found to be attracted by aphids in an olfactometer. For example, Rotheray (1981) observed that parasitoids of syrphid fly larvae were attracted to aphids, thus permitting these parasitoids to locate aphid colonies, the most likely places to find the syrphid hosts. Read et al. (1970) showed that females of the cabbage aphid parasitoid, *Diaeretiella rapae* (McIntosh), responded to hosts freshly removed from collards, but not to aphids removed from host plants 24 hr prior to the test. The authors concluded that attraction to aphids may have resulted from traces of substances from the host plant being still present on test aphids. The attraction of the parasitoid *Lysiphlebus testaceipes* (Cresson) to its host, the greenbug *Schizaphis graminum* (Rondani) (Schuster and Starks, 1974), could be explained similarly. Powell and Zhi-Li (1983) found that males and females of the parasitoids *Aphidius uzbekistanicus* Luzhetski and *A. ervi* Haliday were attracted by odors from the food plants of their aphid hosts, but only females responded to the aphids, which suggests that aphids were attractive owing to their own specific odors. However, attraction to traces of plant substances remaining on aphids cannot be ruled out since the authors found that, at least in the case of *A. uzbekistanicus*, males and females may differ in their reaction to different plant substances. In the case of *A. nigripes*, traces of host plant substances cannot be implicated since parasitoids did not respond to the host plants of the aphids.

Surprisingly, females of our *A. nigripes* strain not only responded to a preferred host, *M. euphorbiae*, but also to the much less preferred hosts *M. persicae* and *A. nasturtii*, and even to a nonhost aphid, *R. maidis*. This suggests that females react nonspecifically to volatiles emanating from various aphid species. Assuming that such general attraction by aphid odors is used to locate hosts over relatively short distances by *A. nigripes*, then host species selection could still be achieved either through proper host habitat selection and/or contact recognition of potential hosts. Then a broad response to nonspecific aphid odors

may still be highly adaptive in enabling searching parasitoids to quickly find aphid colonies dispersed among plants. Females of the two species of aphid parasitoids tested by Powell and Zhi-Li (1983) would not have to rely on host-habitat selection or host recognition to locate the appropriate hosts as they apparently respond only to suitable hosts.

In Petri dishes, *A. nigripes* females did not appear to detect the presence of aphid honeydew from a distance (Bouchard and Cloutier, 1984). However, the findings of the present study clearly demonstrate that females are attracted by honeydew odors carried by an airstream. Thus honeydew, which is a good indicator of the presence of aphids on plants, not only has a contact effect resulting in intensified searching by *A. nigripes* (Bouchard and Cloutier, 1984), but can also attract females from a distance by means of olfaction. Different honeydew constituents are probably responsible for its contact and olfactory effects. For example, the arrestant effect of honeydew on the aphid predator *Chrysopa carnea* Stephens is due to sucrose, whereas volatile breakdown products of tryptophan act as attractants (Hagen et al., 1976; Van Emden and Hagen, 1976). Volatiles would also be implicated in short-range attraction of larvae of the predator *Aphidoletes aphidimyza* (Rond.) to honeydew from its aphid prey (Wilbert 1974).

Female *A. nigripes* did not respond to potato or barley plants in our tests. In *D. rapae*, both sexes were attracted to collards as well as to dilute solutions of allyl isothiocyanate, a mustard oil present in Cruciferae (Read et al., 1970). Similarly *L. testaceipes* responded to odors from sorghum, the food plant of its greenbug host (Schuster and Starks, 1974). The parasitoids *A. uzbekistanicus* and *A. ervi* also showed selective responses to the food plants of their host aphids (Powell and Zhi-Li, 1983). In *A. nigripes*, the absence of reaction to plants might have resulted from rapid changes in the volatile mixture emanating from the plants following cutting (Visser and Nielsen, 1977). An alternative explanation is that *A. nigripes* may use other than olfactory means to locate the habitat of its host, such as visual cues for example. Even though this parasitoid is closely associated with potato fields in North America (Duncan and Couture, 1956; Shands et al., 1965), its host range includes aphids which do not use potatoes as host plant, including *Macrosiphum avenae* Fab. and *M. rosae* L. (Mackauer and Stary, 1967). Moreover, the commonly reported hosts *M. euphorbiae* and *M. persicae* are extremely polyphagous, using host plants not taxonomically restricted to the family level (Eastop, 1977). Thus it is not surprising to find that *A. nigripes* does not seem to use plant volatiles as a means of host-habitat selection, as shown for parasitoids exhibiting greater host and/or habitat specificity such as *D. rapae*. A similar strategy might be inefficient for *A. nigripes*, given the diversity of plant chemicals that it would have to recognize.

*Aphidius nigripes* males were not attracted by aphid odors, which indicates that they do not locate females through the intermediary of aphids. Powell and Zhi-Li (1983) reported similar results for *A. uzbekistanicus* and *A. ervi*. Male

*A. nigripes* did not respond to conspecific males, but were strongly attracted to females. This confirms the observations of Dumas and McNeil (unpublished) indicating the existence of a female sex pheromone in *A. nigripes*, as reported for other aphid parasitoids (Read et al., 1970; Powell and Zhi-Li, 1983). Female *A. nigripes* did not respond to males or females, which suggests that the presence of conspecifics would not interfere with the attractiveness of aphid colonies to searching females.

The present study does not prove long-range attraction of *A. nigripes* to host aphids, but it clearly shows that once in the habitat of potential hosts, the searching female has the capacity to select, via olfactory cues, areas where hosts are most likely to be available. The actual mechanisms used by this parasitoid in host-habitat selection remain to be elucidated, but they are unlikely to implicate olfaction based on volatiles produced by the food plant of the host.

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# TESTS FOR INDUCTION OF FEEDING PREFERENCES IN LARVAE OF EASTERN SPRUCE BUDWORM USING EXTRACTS FROM THREE HOST PLANTS

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**Abstract**—Feeding preferences to extracts from three host plants were determined for sixth-instar eastern spruce budworm larvae reared on one of three hosts or on artificial diet. Preferences of foliage-reared larvae were compared to those of diet-reared, or naive, larvae. No effect of plant induction was found using amino acids and bases and chloroform extracts on test disks in three-choice experiments. Possible effects of habituation for sugars and glycosides from white spruce were shown. Organic acids contributed to a phenomenon of avoidance learning in larvae reared on all three hosts, showing that this fraction from balsam fir was the least deterrent, while that from black spruce was the most deterrent.

**Key Words**—Eastern spruce budworm, Lepidoptera, Tortricidae, *Choristoneura fumiferana*, host preference, deterrence.

## INTRODUCTION

Larvae of the eastern spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae) are oligophagous insects which feed on a variety of coniferous host plants in eastern Canada and the United States. Heron (1965) first studied the responses of the spruce budworm larvae to phytochemicals from its host plants. Albert and Jerrett (1981) showed that the polar fractions of a number of host plants elicit larval feeding and that carbohydrates appeared to be the most effective compounds. Subsequently, it was shown that sucrose was the most stimulating of 12 carbohydrates tested and that the optimal concentration of this chemical was in the range of that which occurs naturally in balsam fir at a time



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of year when larvae were actively feeding on this host in the field (Albert et al., 1982).

In comparing the responses of sixth-instar larvae in feeding tests with extracts from four major host plants, we have shown the strong stimulating effect of the sugars and glycoside fraction from each host (Albert, 1982). Amino acids and bases were only slightly stimulating, and organic acids were either neutral or deterrent depending on the host species tested. Chloroform fractions from balsam fir and black spruce were neutral, while those from red and white spruces were stimulating at concentrations found in the host plants.

In some insects, a preference shown for a particular host plant as compared to other equally suitable hosts can be altered experimentally by rearing the early instar larvae on the less-preferred host, then testing the later instars in a choice situation. When the initially less-eaten host becomes more eaten in such a situation, a preference is said to have been induced for the rearing plant (Jermy et al., 1968). In a recent study, de Boer and Hanson (1984) showed that the ability of a rearing plant to induce a feeding preference in tobacco hornworm larvae depends on the taxonomic relatedness of this plant and its companion test plant. In summarizing the literature on feeding induction, they point out that this phenomenon seems to hold true for most insect species studied. Since taxonomically related plants presumably contain a large number of chemicals which they share in common, it would seem logical to expect that an insect could not easily discriminate between the two host species if the chemosensory information it obtains from each host is the same.

Most previous studies on induction of feeding preferences have used foliage from the host plant as a test substrate. This provides the insect with a complete range of phytochemicals on which to base its feeding decisions. Städler and Hanson (1978) showed that it was possible for a specific chemical to influence the induction of preference in the tobacco hornworm. They also showed that the insect could be induced by nutrients present in the artificial diet on which it was reared. Animals reared on tomato responded differently to hexane, diethyl ether, and methanol extracts of tomato compared to animals reared on wheat germ medium. Accordingly, this paper attempts to determine whether specific groups of phytochemicals could be used by sixth-instar eastern spruce budworm in making feeding decisions after having been reared on foliage from different hosts.

## METHODS AND MATERIALS

*Rearing of Experimental Animals.* Insects were obtained as unfed second instar larvae from the Maritimes Forest Research Center, Fredericton, New Brunswick. They were transferred onto one of four media: artificial diet (McMorran, 1965); balsam fir (*Abies balsamea*) needles; white spruce (*Picea glauca*) needles; or black spruce (*Picea mariana*) needles. All plant materials

were collected at the Acadia Forest Experiment Station, New Brunswick from current-year growth taken on June 17–19, 1980. The fresh foliage was frozen and kept at -18°C for later use in tests.

When larvae reached their late fifth (penultimate) instar, they were deprived of their food and allowed to molt to the sixth instar. These starved sixth-instar larvae were used in the feeding tests. Insects developed normally on the artificial diet, balsam fir, and white spruce. However, mortality was greater than 50% for those reared on black spruce foliage.

*Feeding Preference Tests*. For feeding experiments, an individual larva was placed in a test arena as described previously (Albert et al., 1982). Cellulose filter disks were pinned 2 mm above the floor of a styrofoam sheet, arranged in a circular fashion for three-choice tests (a-b-c-a-b-c, etc.). Nine disks were used in each test arena, which was covered by a 3.5-cm-diam. by 1-cm-high Petri dish. Disks were wetted with a 15-$\mu$l aliquot of the chemical solution (plant extract) to be tested. For tests using chloroform extracts, the disks were wetted with 15 $\mu$l of the extract, the chloroform was allowed to evaporate, then the disks were wetted with 15 $\mu$l of distilled water. All host-plant extracts were tested at a concentration equivalent to that found in the host plant at the time of its collection in the field.

Animals were allowed to feed on disks for 24 hr, except for tests with organic acid extracts which lasted 48 hr. Animals which ate less than 50% or greater than 250% of the total disk area available (max. 300% possible) for the preferred group of disks were discarded. Results were analyzed with the simultaneous test procedure (STP), with a significance level for rejection of the null hypothesis set at $P = 0.05$ (Sokal and Rohlf, 1969).

*Plant Extractions*. Freeze-dried samples of the same lot of plants collected for rearings were extracted with methanol, followed by column chromatography on Amberlite resins (Albert and Jerrett, 1981). The following four fractions were recovered from each host: sugar and glycoside; amino acid and base; organic acid; and chloroform extract (lipids, organic esters, alcohols, aldehydes, waxes, etc.). Based on their weight, and the percentage of moisture content of the foliage collected, these extracts were redissolved in a volume of distilled water that resulted in a final concentration of the solution which was equal to the concentration in each plant.

## RESULTS AND DISCUSSION

*Tests with Sugar and Glycoside Extracts*. Animals reared on diet preferred the sugar and glycoside extracts of balsam fir and black spruce over those of white spruce (Figure 1). This preference was reversed for animals reared on balsam fir foliage. However, when reared on white spruce foliage, larvae did not discriminate between balsam fir and either white spruce or black spruce,

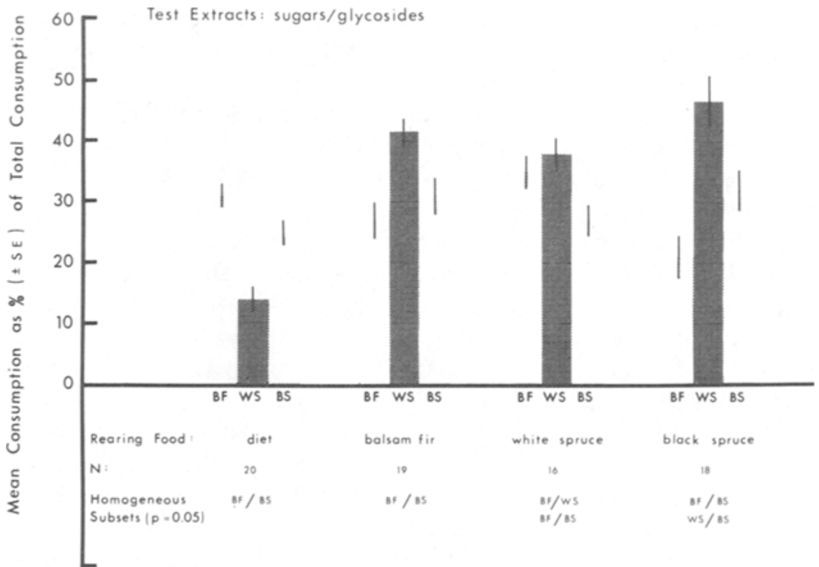


FIG. 1. Results of three-choice tests showing feeding preferences for sugar and glycoside extracts of balsam fir (BF), white spruce (WS), and black spruce (BS) for animals reared on diet, balsam fir, white spruce, or black spruce. $N$ = number of experimental animals.

but maintained a preference for white spruce over black spruce. When reared on black spruce, there was a subsequent preference for white spruce over balsam fir, but no discrimination was found between balsam fir and black spruce, or between white spruce and black spruce.

The importance of sugars, especially sucrose, has been shown previously for spruce budworm larvae (Albert et al., 1982; Albert, 1982). The present strong overall preference for the white spruce sugar and glycoside extract is consistent with the findings of the previous studies. There appears to be some subtle effect of rearing experience on later feeding preferences. How this relates to the current concepts of induction will be addressed later in this section.

*Tests with Amino Acid and Base Extracts.* A strong feeding preference for amino acids and bases from black spruce foliage is seen irrespective of which of the four foods was used to rear the animals (Figure 2). That black spruce amino acids are preferred is surprising, especially in light of the fact that animal populations reared on this plant showed more than 50% mortality (cf. Methods and Materials). It is also commonly accepted that spruce budworm larvae tend to avoid black spruce in the field when either balsam fir or white spruce are available in the same area. The results with amino acid and base extracts suggest that black spruce contains stimulative components in this fraction and that the

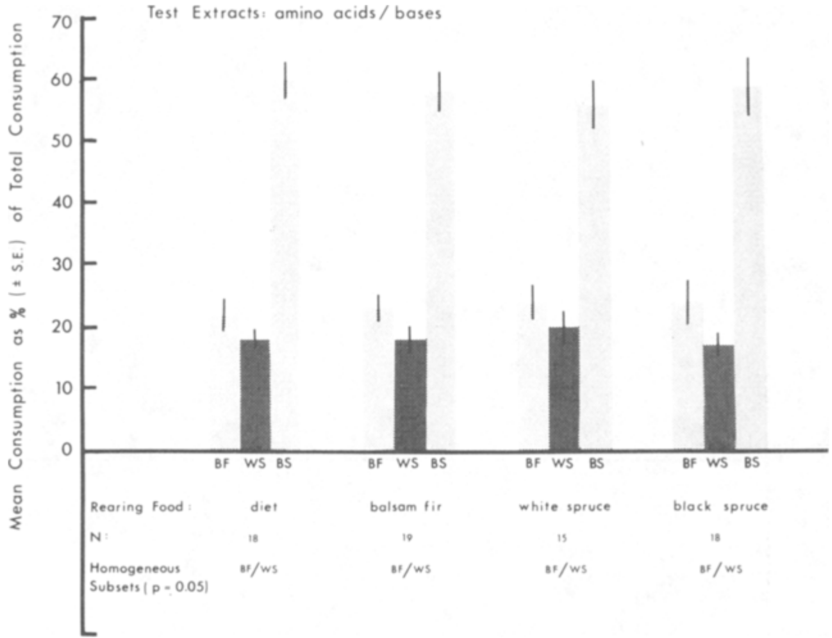


FIG. 2. Results of three-choice tests showing feeding preferences for amino acid and base extracts of balsam fir (BF), white spruce (WS), and black spruce (BS) for animals reared on diet, balsam fir, white spruce, or black spruce. $N$ = number of experimental animals.

lower preference shown for its foliage is more likely due to the presence of deterrents. Amino acids and bases from balsam fir and those from white spruce were equally stimulating (Figure 2).

*Tests with Organic Acid Extracts.* Organic acids extracted from any of the three hosts are either neutral or deterrent in two-choice feeding tests using water as the control chemical (Albert, 1982). Diet-reared animals showed no preference for any of the organic acid extracts (Figure 3). When reared on balsam fir, larvae showed a slight preference for the balsam fir organic acids, although this was not statistically significant (at $P$ = 0.05). When reared on either white or black spruce foliage, larvae showed a significant preference for the organic acids from balsam fir. This is a very interesting phenomenon, since this extract from balsam fir has been shown previously to be deterrent relative to the water control (Albert, 1982). We interpret this to signify that, in circumstances where animals are forced to feed on organic acids, those from balsam fir are the least deterrent of the three. Rearing larvae on white spruce and black spruce may thus have the effect of sensitizing the animals to their organic acids, a process of "negative

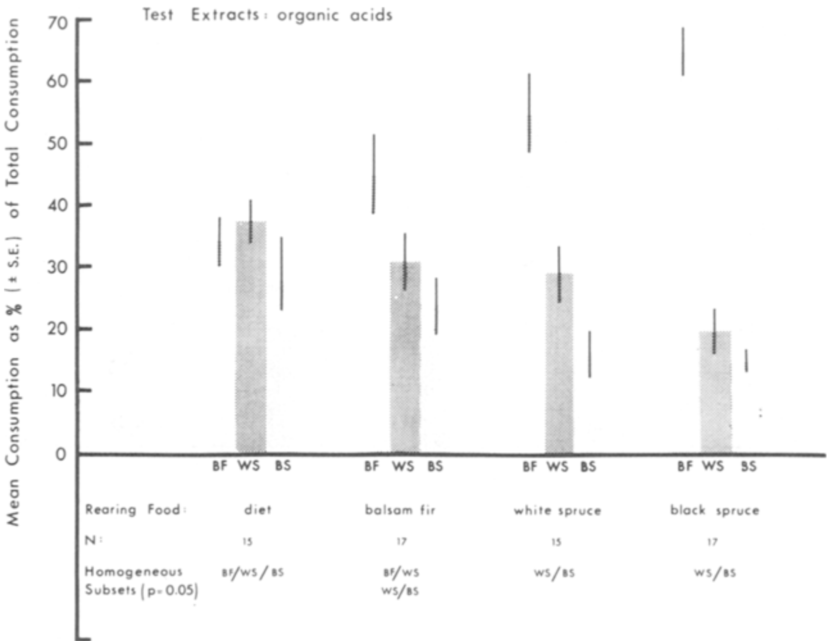


FIG 3. Results of three-choice tests showing feeding preferences for organic acid extracts of balsam fir (BF), white spruce (WS), and black spruce (BS) for animals reared on diet, balsam fir, white spruce, or black spruce. $N$ = number of experimental animals.

induction," which is later seen as a shift to preference for the least deterrent balsam fir organic acids.

*Tests with Chloroform Extracts*. Animals reared on any of the food sources consistently showed an enormous preference for the chloroform fraction from white spruce (Figure 4). The stimulating effect of this fraction was discussed previously (Albert, 1982). It undoubtedly contains one or more very strong feeding stimulants. In two-choice tests, animals exhibited the strongest preference overall for this particular fraction.

*Comparisons*. Results of our experiments show that the three host plants studied have no effect of inducing early instars when the amino acids and bases or the chloroform extracts are used as a measure of their inductive properties. When sugar and glycoside extracts were used to determine whether induction had occurred, the only effect we measured was a loss of discrimination between balsam fir and white spruce for animals reared on white spruce. Similarly, we measured a loss of discrimination between white spruce and black spruce for animals reared on black spruce. Rearing on white spruce may have had the effect of habituating larvae to its sugars and glycosides. Animals reared on black spruce

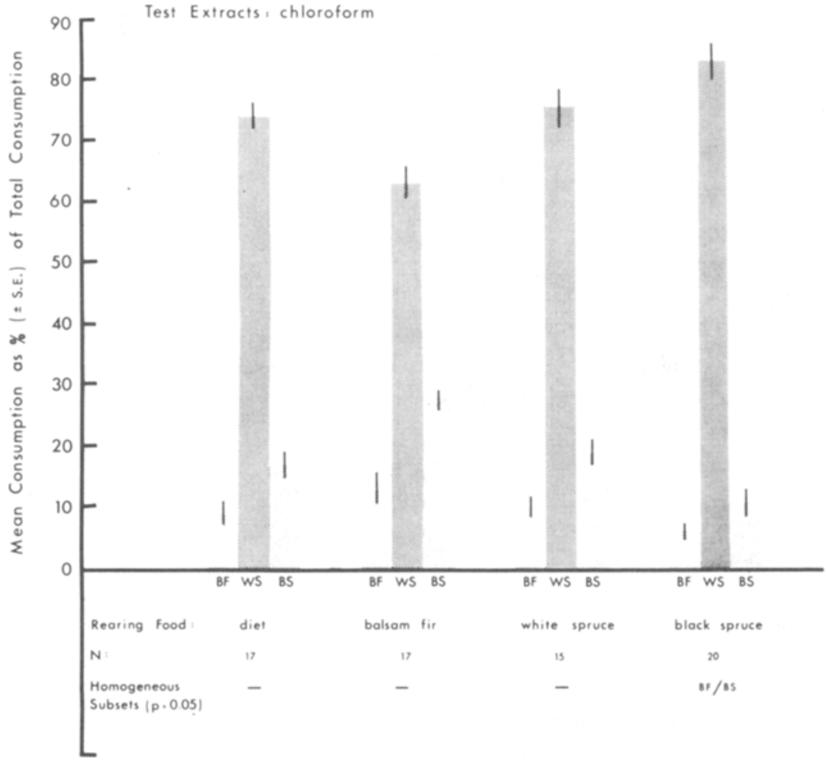


FIG. 4 Results of three-choice tests showing feeding preferences for chloroform extracts of balsam fir (BF), white spruce (WS), and black spruce (BS) for animals reared on diet, balsam fir, white spruce, or black spruce. $N$ = number of experimental animals.

later exhibited a return to a general preference for white spruce, but lost the ability to discriminate between sugar and glycoside extracts from white spruce and those from black spruce.

It is with organic acids that the most dramatic effect of a food source inductive effect was seen. It is known that, of the extracts of polar compounds found in the host plants studied, organic acids are the only ones with a feeding deterrent effect (Albert, 1982). In a situation when diet-reared (naive) larvae were forced to choose between deterrent substances from three hosts, they fed equally on all three. However, rearing on balsam fir resulted in an overall preference for its organic acids, suggesting that these are the least deterrent of the group. When reared on white spruce, the same pattern of response was found, with a slight increase in the preference for balsam fir organic acids. Black spruce-reared animals later showed the greatest shift to a relative preference for balsam

fir organic acids, which suggests that its organic acids are the most deterrent of the group, while emphasizing that balsam firs are the least deterrent. This indicates that there has been some form of avoidance learning in the larvae, a phenomenon which has been discussed by Dethier (1982).

In most previous reports of feeding induction by a host plant, whole leaves have been used to test the "induced" animals. It has been shown that polyphagous insects are often easily induced by distantly related plants. Oligophagous insects, however, which can only be tested with closely related plant species, tend to be induced only weakly or not at all by closely related plants (DeBoer and Hanson, 1984). In our experiments, it is clear that certain groups of chemicals, such as the normally deterrent organic acids, can have the effect of inducing the animals, possibly through aversion learning in the present situation. Other groups of chemicals, such as the very stimulating sugars, can have more subtle effects in modifying the feeding preference of the insect, possibly through the phenomenon of habituation, as seen with the sugar and glycoside fractions. Sugars from white spruce were preferred by foliage-reared animals, except for those reared on that host.

Although amino acid and base fractions were consistently chosen in the same preference ratios, thereby showing that none of the three hosts was capable of inducing the animals with these chemicals, the results nevertheless point to a possibly very important role for them in the insect's feeding behavior. This fraction from black spruce is clearly more stimulating than the same fraction from the other two hosts. It may compensate for the more deterrent organic acids fraction from black spruce, by contributing important neural information to the positive side of the input channels to the central nervous system, as explained in Dethier's hypothesis (Dethier, 1982). The chloroform-soluble components of the host plants would presumably also contribute to the total sensory information in the same way, and perhaps more strongly so.

The oligophagous eastern spruce budworm larva, like the tobacco hornworm larva (Städler and Hanson, 1978), shows the ability to discriminate between various chemical components from its host plants and uses information obtained from these chemicals in its feeding decisions. Different chemical classes have differing degrees of importance in establishing the final decision taken by the insect. Whether individual components of the extracted fractions can achieve the same degree of influence on the central decision-making processes remains to be investigated.

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# ELECTROANTENNOGRAM RESPONSES OF GRAPE BORER *Xylotrechus pyrrhoderus* BATES (COLEOPTERA: CERAMBYCIDAE) TO ITS MALE SEX PHEROMONE COMPONENTS

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(Received June 27, 1984; accepted October 9, 1984)

**Abstract**—Electroantennograms were recorded from the grape borer *Xylotrechus pyrrhoderus* in response to serial dilutions of male sex pheromone components, (2*S*,3*S*)-octanediol and (2*S*)-hydroxy-3-octanone, and to 100 μg of their optical isomers and host plant substances. Female antennae always responded more strongly than male antennae. Antennae of both sexes were highly sensitive to (2*S*)-hydroxy-3-octanone. F/M ratio (female to male EAG value) was greater for male sex pheromone components, especially (2*S*,3*S*)-octanediol, and their optical isomers than plant substances. Antennal sensitivity to optical isomers (2*R*,3*R*-octanediol and 2*S*,3*R*-octanediol) was lower than true pheromone components.

**Key Words**—Electroantennogram, grape borer, *Xylotrechus pyrrhoderus* Bates, Coleoptera, Cerambycidae, male sex pheromone, (2*S*,3*S*)-octanediol, (2*S*)-hydroxy-3-octanone, optical isomers.

## INTRODUCTION

The grape borer *Xylotrechus pyrrhoderus* Bates is a major pest of grapevines in Japan. The courtship behavior involves a male sex pheromone attractive to females over a distance of 1–1.5 m (Iwabuchi, 1982).

The male sex pheromone was identified as a two-component mixture of (2*S*,3*S*)-octanediol (*S*,*S*-diol) and (2*S*)-hydroxy-3-octanone (*S*-ketol), although the detail of their biological significance has not been clarified (Sakai et al., 1984).



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The electroantennogram (EAG) technique has proved to be useful for identifying the primary pheromone component, particularly in lepidopterous species, and for obtaining valuable information on the coleopterous antennal perception of pheromones for which the behavioral response is known.

The present study was carried out to clarify the antennal responses of the grape borer to synthetic male sex pheromone components and to compare them with responses to their optical isomers and other plant substances.

Nishino et al. (1977) proposed a M/F ratio, the value of male mean response divided by the female mean response. This ratio reflects the patterns of perception of stimuli to the olfactory receptors and represents the discrimination between sexually active compounds and general odorous compounds (Nishino et al., 1977). It has been suggested (Nishino et al., 1980) that a high value would occur if the female antenna has many receptors for an odorant (e.g., sex pheromone) while the male antenna has few or none. Instead of the M/F ratio, we propose the F/M ratio for the grape borer, since the grape borer's pheromone was male-produced and it is the female which responds to the pheromone.

## METHODS AND MATERIALS

*Animals.* Pupae taken from vine cuttings were sexed and maintained in the laboratory under a photoperiod of 14 hr light–10 hr dark at 25°C. Adults of both sexes were reared separately in glass tubes (1.5 × 3.5 cm) for a period of 15 days, the period required for sexual maturation (Iwabuchi, 1982), before being used for EAG tests. To determine the effect of age on the EAG response, 1-, 7-, 20-, and 25-day-old females were also tested.

*Stimulating Chemicals.* The grape leaf volatile compounds were identified by Wildenradt et al. (1975). Of the major compounds of grape leaf volatile, the following were tested for EAG experiments: hexanol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, citral, linalool, (*E*)-2-hexenal, and geraniol. In addition, eugenol, which is not reported as a grape leaf volatile compound, was also tested. Chemicals known as major components of vine leaves were obtained commercially.

Pheromone components, *S*,*S*-diol and *S*-ketol, and their isomers were synthesized according to the procedure reported by Sakai et al. (1984). That is, *S*,*S*-diol and its optical isomers (*R*,*R*-, *S*,*R*-, and *R*,*S*-diols) were obtained from methyl D- or L-tartrate according to the synthesis of exobrevicomin by Mori et al. (1974), while *S*-ketol and its optical isomer (*R*-ketol) were prepared, respectively, from L-lactic acid and from D-alanine treated with amyllithium. Physical data of the compounds are as follows:

(2S,3S)-*Octanediol* (S,S-*diol*). $[\alpha]_D^{23}$ −19.2°; [$^1$H]NMR, $\delta$ 0.90 (3H, t, $J$ = 6.7 Hz), 1.19 (3H, d, $J$ = 6.3 Hz), 2.01 (1H, d, $J$ = 5.9 Hz), 2.07 (1H, d, $J$ = 5.9 Hz), 3.33 (1H, m), 3.59 (1H,m); MS, $m/z$ 101 (6%, $M^+ - CH_3CHOH$), 83(59), 75(4), 57(15), 55(100), 45(22), 43(22), 41(24).

(2R,3R)-*Octanediol* (R,R-*diol*). $[\alpha]_D^{27}$ +17.5°.

(2S,3R)-*Octanediol* (S,R-*diol*). mp 62–64°; $[\alpha]_D^{27}$ +17.0°; [$^1$H]NMR, $\delta$ 0.90 (3H, t, $J$ = 6.0 Hz), 1.14 (3H, d, $J$ = 7.0 Hz), 3.60 (1H, m), 3.77 (1H, m); MS, $m/z$ 101 (6%, $M^+ - CH_3CHOH$), 83(59), 75 (4), 57(15), 55(100), 45(22), 43(22), 41(24).

(2R,3S)-*Octanediol* (R,S-*diol*). mp 60–63°; $[\alpha]_D^{27}$ −18.3°.

(2S)-*Hydroxy-3-octanone* (S-*ketol*). $[\alpha]_D^{24}$ +51.8°; [$^1$H]NMR, $\delta$ 0.90 (3H, t, $J$ = 6.0 Hz), 1.38 (3H, d, $J$ = 7.0 Hz), 4.23 (1H, dq, $J$ = 5, 6 Hz); MS, $m/z$ 101 (1%, $M^+ - CH_3CO$), 99(22), 83(6), 71(25), 55(28), 45(51), 43(100), 41(13).

(2R)-*Hydroxy-3-octanone* (R-*ketol*). $[\alpha]_D^{25}$ −54.4°.

The chemical purity of these compounds was 99% or greater, as determined by GLC and NMR analysis. Serial dilutions of the compounds were made in purified hexane and stored at −15°C until used.

HO OH

(2*S*,3*S*)-Octanediol
(*S*,*S*-diol)

O OH

(2*S*)-Hydroxy-3-octanone
(*S*-ketol)

*EAG Recording.* EAGs were recorded from excised antennae of adult male and female grape borers according to the modified EAG technique used by Roelofs and Comeau (1971). The filter paper disk impregnated with the test compound was placed on a vinyl tubing holder which had been positioned in a 10-ml syringe. The syringe plunger was quickly pushed to mix the compound into a continuous air stream (13.3 ml/sec), and the air containing the compound was puffed onto the fixed antennal preparation through 0.5-cm-diameter glass tubing. The tubing was terminated at 1.0 cm from the distal and middle parts of the antenna. Stimulation of the antennal preparation with hexanol, *S*,*S*-diol, and *S*-ketol was performed in this sequence from the lowest to the highest concentrations. Optical isomers (100 $\mu$g) were presented to each antenna in random order. All antennal preparations were also exposed to a hexane control and a standard of 100 $\mu$g hexanol. Responses were standardized by subtracting the control value (0.20 ± 0.03 mV for females, 0.11 ± 0.02 mV for males) and expressing the response to pheromone components and their optical isomers as a percentage of the response to the standard, 100 $\mu$g hexanol. The data were compared with the least-significant-difference test. At least one minute was allowed between each stimulus. This interval was found to be adequate for recovery of the EAG. Fifteen replicates were recorded from each sex to each stimulus.

## RESULTS AND DISCUSSION

*Antennal Responsiveness to Plant Substances.* The antennae of both sexes responded more to hexanol and (*E*)-2-hexen-1-ol than to others (Table 1). Dose–

Table 1. EAG Responses(mV)[a] of Grape Borer to Plant Substances

| Substance (100 μg) | Female | Male | F/M ratio[b] |
|---|---|---|---|
| Hexanol | 0.78 ± 0.09 a | 0.41 ± 0.05 ab | 1.9 |
| (*E*)-2-Hexen-1-ol | 0.71 ± 0.07 a | 0.46 ± 0.07 a | 1.6 |
| (*Z*)-3-Hexen-1-ol | 0.37 ± 0.07 b | 0.17 ± 0.02 c | 2.2 |
| Eugenol | 0.33 ± 0.04 b | 0.26 ± 0.03 bc | 1.3 |
| Citral | 0.30 ± 0.05 b | 0.20 ± 0.03 c | 1.5 |
| Linalool | 0.24 ± 0.05 b | 0.11 ± 0.01 c | 2.3 |
| (*E*)-2-Hexenal | 0.23 ± 0.04 b | 0.15 ± 0.02 c | 1.6 |
| Geraniol | 0.21 ± 0.03 b | 0.12 ± 0.03 c | 1.7 |

[a] X ± SE of 15 tests. Values in a column not followed by the same letters are significantly different at $P < 0.05$.
[b] Female mean response divided by male mean response.

EAG response curves to hexanol showed an increase in the amplitude of the EAG in the range of 10 μg–10 mg of hexanol without reaching a plateau, but 1 μg–10 μg amounts were essentially inactive (Figure 1). The difference of EAG response between the sexes was greater at higher concentrations. EAG responses to the plant substances (100 μg) tested resulted in F/M ratios ranging from 1.3 to 2.3. The size of the antenna and the number of receptors reflect upon the antennal sensitivity (Seabrook, 1978). The tribe Clytini, to which the grape borer belongs, is characterized by short antennae. The sizes of the female and male antennae of the grape borer were 4.8 mm and 5.4 mm long, respectively. Since the sexual dimorphism in antennal size does not seem to be sufficient to explain the difference of antennal sensitivity for each sex, there may be differences in the numbers of receptors for plant substances, probably sensilla basiconica, which are known as receptor sites for plant substances in *Monochamus* species (Dyer and Seabrook, 1975, 1978).

*Antennal Responsiveness to Male Sex Pheromone.* Relative antennal sensitivity showed that *S*-ketol elicited higher EAG responses in both sexes then *S*,*S*-diol or hexanol and that the EAG response to *S*,*S*-diol was slightly higher than hexanol only in females (Figure 1). It was suggested that the high sensitivity to lower concentrations of *S*-ketol allows the female to orient to the pheromone source from a distance. The responses of female antennae to *S*,*S*-diol and *S*-ketol were much higher over the entire range of concentrations than those of males. The sexual specificity in antennal sensitivity was greater for *S*,*S*-diol than for *S*-ketol, and the F/M ratio was 4.6 for *S*,*S*-diol and 3.2 for *S*-ketol (Table 2). The values of the F/M ratio for pheromone components were larger than for plant substances. This suggested there are specific receptors for pheromone components.

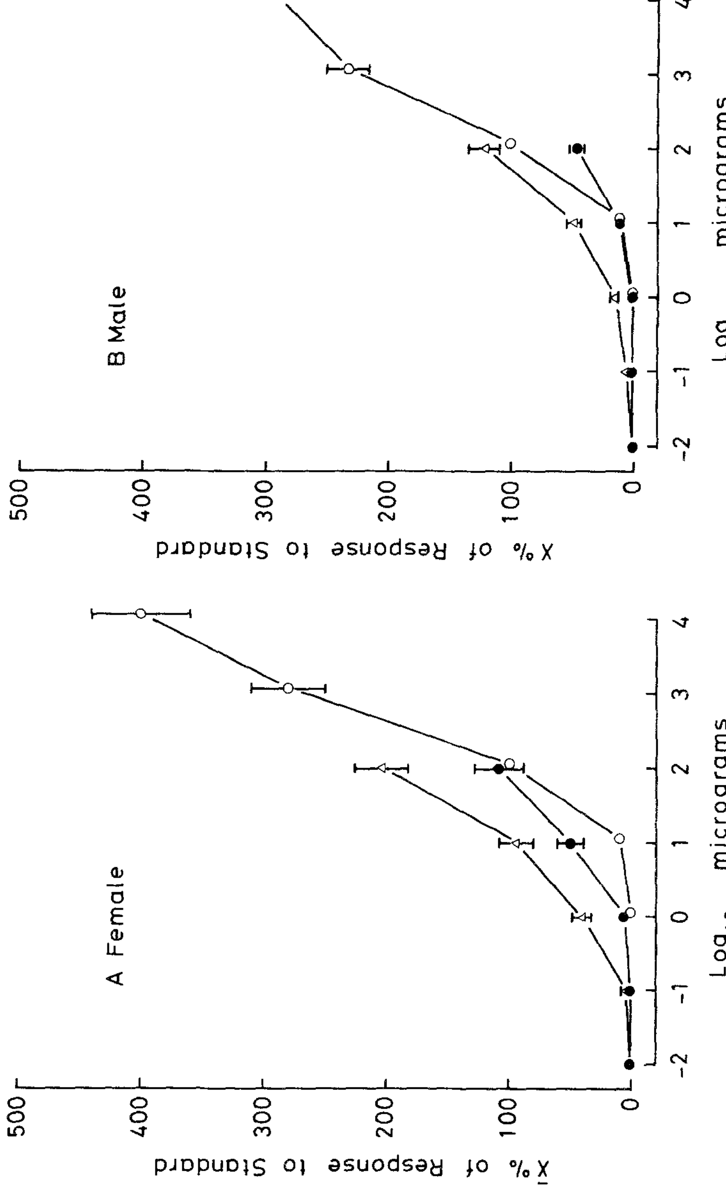


FIG. 1. Dose–EAG response curves of females (A) and males (B) of the grape borer to hexanol (○), (2*S*,3*S*)-octanediol (●), and (2*S*)-hydroxy-3-octanone (△).

Table 2. EAG Responses[a] of Grape Borer to Synthetic Male Sex Pheromone [(2*S*,3*S*)-Octanediol and (2*S*)-Hydroxy-3-octanone] and Its Optical Isomers.

| Substance (100 μg) | Female | Male | F/M ratio[b] |
|---|---|---|---|
| *S*,*S*-Diol | 106.78 ± 20.45 b | 44.00 ± 6.31 bc | 4.6 |
| *R*,*R*-Diol | 47.47 ± 9.13 c | 19.57 ± 6.30 cd | 4.6 |
| *S*,*R*-Diol | 57.94 ± 9.41 c | 16.67 ± 4.17 d | 6.6 |
| *R*,*S*-Diol | 13.67 ± 7.28 c | 12.44 ± 6.85 d | 2.1 |
| *S*-Ketol | 203.79 ± 23.57 a | 120.00 ± 12.90 a | 3.2 |
| *R*-Ketol | 102.98 ± 17.19 a | 52.45 ± 14.63 b | 3.7 |

[a]Responses are mean percentages ($X$ ± SE of 15 tests) to a standard of 100 μg hexanol. Values in columns not followed by the same letters are significantly different at $P < 0.05$ (LSD).
[b]Female mean response divided by male response.

Sexual specificity in antennal sensitivity is generally observed in responses of lepidopterous species to their female sex pheromone; in such cases, the amplitude of the EAG responses obtained from male antennae is much greater than that from female antennae, because the female lacks the sensilla trichodea which are sensitive to the female sex pheromone (Seabrook, 1978). The greater sensitivity of the male antennae to the female sex pheromone was also reported for other groups of insect (Boeckh et al., 1970; Nishino et al., 1977; Chuman et al., 1982). On the other hand, there is no difference in the antennal sensitivity of the sexes in the species which use an aggregation pheromone (Payne, 1971; Gutmann et al., 1981; Grant and Lanier, 1982; Dickens et al., 1983; Light, 1983). Although little is known about the antennal sensitivity to male sex pheromone, except for the cases of aphrodisiac substances of lepidopterous species (Grant, 1971; Grant et al., 1972; Seibt et al., 1972; Payne and Finn, 1977), these facts suggested that female predominance in the antennal sensitivity to the plant substances and male sex pheromone was characteristic of insect species with male sex pheromone attractive to females over a distance. Although the behavioral significance is not necessarily reflected by EAG characteristics, the sexual difference in the antennal sensitivity of the grape borer to its male sex pheromone, especially *S*,*S*-diol, may be an advantage for the female's perception of pheromone molecules.

*Antennal Responsiveness to Optical Isomers.* The grape borer responded to the optical isomers of *S*,*S*-diol and *S*-ketol, but the EAG amplitudes were significantly lower than the true pheromone components (Table 2). *R*,*R*-Diol and *R*-ketol seem to be perceived by the same system, probably the same receptors, as their true pheromone components, since F/M ratios for them were similar to the true pheromone components. However, *S*,*R*-diol and *R*,*S*-diol had different

characteristics and may be perceived by a different system from other isomers, because of the higher or lower F/M ratio indices. Pheromone receptors generally respond not only to a single compound but to a range of compounds with structural similarities (Seabrook, 1978; Nishino et al., 1980; Payne et al., 1973, 1982). For example, pheromone receptors respond to the isomers and analogs of the appropriate pheromone, although the response is weaker than to the true pheromone (Schneider et al., 1964; Payne, 1971). In the case of bombykol, the geometric isomers were up to 1000 times less effective at the bombykol receptor than the true bombykol (Schneider et al., 1964). Similar cases were also reported for the pheromone analogs of boll weevils (Gutmann et al., 1981) and the enantiomeric components of the aggregation pheromone of *Ips paraconfusus* (Light, 1983).

*Effect of Age of Females on Their Antennal Sensitivity.* The EAG amplitudes of the female grape borer to 100 μg of hexanol, *S*,*S*-diol, and *S*-ketol increased with time after adult eclosion. However, the age did not influence the relative sensitivity (Figure 2). In general, the antennal response to sex phero-

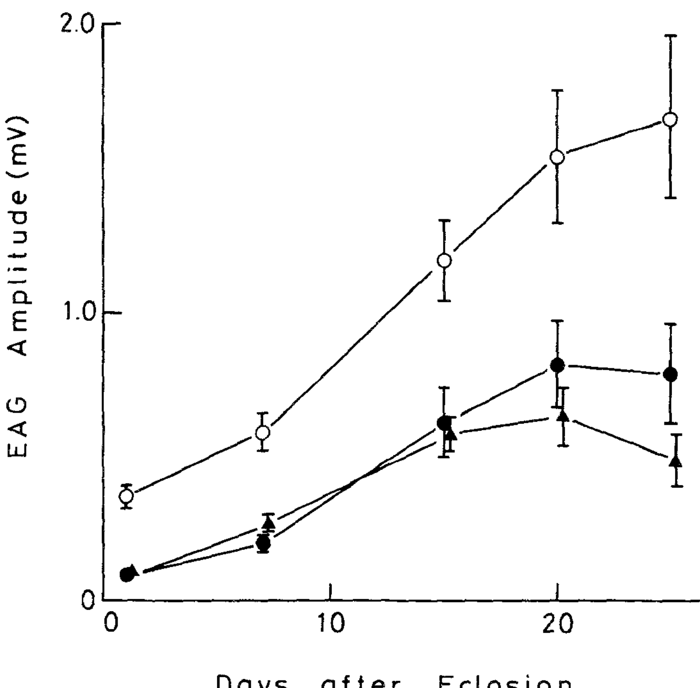


FIG. 2. Effect of age on response to 100 μg hexanol (▲), 100 μg (2*S*,3*S*)-octanediol (●), and 100 μg (2S)-hydroxy-3-octanone (○). Vertical lines represent ±SE. Points are means of recordings from 15 beetles for day 0–20, and 10 beetles for day 25.

Table 3. Response Time (A), Recovery Rate (B) and Recovery Index (C) of EAG Responses for Grape Borer to Synthetic Male Sex Pheromone and Its Optical Isomers[a]

| Substance (100 $\mu$g) | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | A | B | C | A | B | C |
| *S,S*-Diol | 0.69 ± 0.02 c | 0.85 ± 0.09 c | 1.21 ± 0.11 a | 0.67 ± 0.05 c | 0.93 ± 0.11 bc | 1.37 ± 0.11 a |
| *R,R*-Diol | 0.66 ± 0.03 c | 0.66 ± 0.03 d | 1.02 ± 0.07 a | 0.63 ± 0.69 cde | 0.69 ± 0.08 cd | 1.09 ± 0.13 a |
| *S,R*-Diol | 0.64 ± 0.03 c | 0.69 ± 0.03 cd | 1.10 ± 0.07 a | 0.50 ± 0.02 e | 0.67 ± 0.07 d | 1.37 ± 0.16 a |
| *R,S*-Diol | 0.72 ± 0.04 c | 0.81 ± 0.05 cd | 1.14 ± 0.07 a | 0.65 ± 0.04 cd | 0.66 ± 0.07 d | 1.06 ± 0.15 a |
| *S*-Ketol | 1.22 ± 0.08 a | 1.57 ± 0.10 a | 1.30 ± 0.05 a | 1.15 ± 0.06 a | 1.45 ± 0.08 a | 1.27 ± 0.06 a |
| *R*-Ketol | 0.93 ± 0.04 b | 1.26 ± 0.05 b | 1.39 ± 0.10 a | 0.91 ± 0.04 b | 1.14 ± 0.09 b | 1.26 ± 0.10 a |

[a]Values represent mean percentages ($X \pm$ SE) of 7–15 tests to a standard of 100 $\mu$g hexanol. Mean values for 100 $\mu$g hexanol: female A = 0.5 sec; B = 1.28 sec; C = 2.59. Male A = 0.54 sec; B = 1.40 sec; C = 2.62. Values in a column not followed by the same letters are significantly different at $P < 0.05$.

mone is independent of adult age (Seabrook, 1977). But some reports demonstrated that the antennal sensitivity to the sex pheromone changed with adult ages (Roelofs and Comeau, 1971; Skirkyavichus and Skirkyavichene, 1978, 1979). Seabrook et al. (1979) also reported the EAG response of *Pseudaletia unipuncnnta* to the male pheromone varies with age. In the grape borer, the adult eclosion occurs in vine shoots and adults stay there for about two weeks. During this period, melanization of body surface and development of ovaries occur. Development of the olfactory receptors on antennae may also accompany the entire physiological and morphological changes associated with age.

*Response Time, Recovery Rate, and Recovery Index.* Response time (the time required to attain maximum amplitude) and recovery rate (the time to return halfway to the base line) were smaller for 100 $\mu$g of optical isomers of diol than those of ketol, at least for females. However, these values were not apparently different among diol isomers, while, for both sexes, these values for *R*-ketol were smaller than those for *S*-ketol. On the other hand, there were no differences among recovery indices (the values of recovery rate divided with the response time) for all optical isomers of diol and ketol (Table 3). These facts suggested that similar systems to bind the odorant molecule to the receptor and to deactivate the molecule exist for diols.

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# THREE EUROPEAN CORN BORER POPULATIONS IN NEW YORK BASED ON SEX PHEROMONES AND VOLTINISM

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**Abstract**—Pheromone blend analyses of glands from individual female European corn borers, *Ostrinia nubilalis* (Hübner), from field-collected larvae or pupae associated with bivoltine flights in June and August and a univoltine flight in July have shown that: (1) a site in western New York has a bivoltine biotype utilizing (*Z*)-11-tetradecenyl acetate as its primary pheromone component (designated *Z*), (2) two sites in central New York have mixed populations consisting of a bivoltine biotype utilizing the *E* pheromone isomer (designated *E*) and a univoltine biotype utilizing *Z*, and (3) one site in central New York was found to have only the univoltine *Z* biotype. The combinations of voltine biotypes and pheromone strains found in New York support the existence of three European corn borer populations designated bivoltine *E*, bivoltine *Z*, and univoltine *Z*.

**Key Words**—European corn borer, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, voltinism, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, pheromone.

## INTRODUCTION

Distinct European corn borer (ECB), *Ostrinia nubilalis* (Hübner), populations in New York have been documented since their first detection around 1920. Apparently this pest was introduced with shipments of broomcorn from central Europe and Italy to several centers of infestation (Caffrey and Worthley, 1927). The initial infestation in New York was univoltine (Hervey and Carruth, 1939),

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and by 1939 had spread westward around the Great Lakes through Pennsylvania, Ohio, Michigan, Indiana, eastern Wisconsin, and into Ontario, Canada. A bivoltine biotype was found in eastern Massachusetts, and by 1939 it had spread westward to New York and had heavily infested the Long Island and Hudson River Valley areas. The bivoltine biotype was found to feed upon ca. 200 different plants, whereas the univoltine biotype at that time appeared to infest mainly broomcorn and corn (Caffrey and Worthley, 1927).

By 1943 (Carruth, 1943), a bivoltine biotype had been noted in all of New York. The univoltine biotype, however, remained dominant in central and western New York until the mid-1960s when the bivoltine biotype predominated (McEwen et al., 1968). Black-light trap catches in central New York (site near Geneva) from 1967 to 1976 showed typical bivoltine activity, with distinct flights in June and August (Eckenrode et al., 1983). However, starting in 1977, increasing numbers of moths were captured in July, suggesting an increase in the univoltine biotype in the Geneva area.

Intense monitoring with black-light traps in 28 locations in central and western New York in 1981 revealed that 16 of the sites exhibited a three-flight pattern (bivoltine plus univoltine), whereas some sites had flights only in June and August (bivoltine), and the remaining sites had a single flight in July (univoltine) (Eckenrode et al., 1983). A summary of trap counts from later surveys (unpublished data[2]) suggests that mixed populations continue to occur at most sites (e.g., King Ferry, New York), except for a bivoltine population predominating in western New York in Eden and a univoltine population in central New York State at Paris.

In addition to variable voltinism, ECB populations have been found with two distinct sex pheromone communication systems: a Z strain using a 97:3 mix of (*Z*)- and (*E*)-11-tetradecenyl acetates (Z11–14:OAc and *E*11–14:OAc) (Klun et al., 1973), and an *E* strain using a 4:96 blend of *Z*/*E*11–14:OAcs (Kochansky et al., 1975). The *Z* strain is widely distributed, representing nearly all of the populations surveyed in Europe as well as in North America (Klun, 1975), whereas the *E* strain was found primarily in the northeastern United States and in Italy. Allozyme data suggest genetic divergence between the two strains (Harrison and Vawter, 1977; Cardé et al., 1978; Cianchi et al., 1980). The present study was initiated to correlate pheromone strains with voltinism biotypes at selected geographic sites representing bivoltine, univoltine, or mixed populations. Although pheromone analysis of female moths taken from black-light traps throughout each flight period would appear to be a possible approach to this study, we found that the females (probably mated) contained pheromone contents below our level of detection. In this paper, populations differing in voltinism are referred to as biotypes, whereas those differing in pheromone blends are referred to as strains.

[2] IPM Programs, NYSAES, Geneva, New York 14456.

## METHODS AND MATERIALS

*Rearing.* Field-collected eggs, larvae, and/or pupae associated with each flight peak (>5% of total season's catch) occurring at Eden, Geneva, King Ferry, and Paris, as determined by black-light trap catches, were mass reared in round plastic dishes (25.6 × 9.75 cm) held at 30°C with a 16-hr photophase. These dishes were modified (Reed et al., 1972) to include a 5.6-cm hole in the top covered with screen mesh small enough to contain first instar ECB but still permitting exchange of air and water vapor. Tops of the dishes and a 3.8-cm strip on the sides were painted black to encourage larvae to pupate in the top of the dish on paper toweling. The wheat germ diet was similar to one described by Reed et al. (1972), except that 75% less sorbic acid was used in this study and aureomycin, methyl paraben, propionic acid, formaldehyde, and fumidil B were included.

In 1983 and 1984, newly emerged, unmated adults also were obtained from infested corn stubble placed in 1.1 × 1.1 × 4-m field cages in May or early June. Bivoltine moths emerged from this stubble in June, and univoltine moths emerged from the same stubble in July. Second generation bivoltine moths were obtained from field-collected pupae in August just prior to emergence since diapausing univoltine individuals remain as larvae until the following year.

*Analysis of Sex Pheromone Blends.* Individual female sex pheromone glands were analyzed with a modified procedure reported by Klun and Maini (1979). Adult females were placed individually in 3-oz plastic cups on the day of emergence with a small dental wick soaked with water. The cups were wrapped with aluminum foil at 4 PM on day 1 and the moths kept in total darkness until their sex pheromone gland was excised the next morning at ca. 10 AM. The pheromone gland was removed by snipping off the abdominal tip just anterior to the ring gland. The tip was transferred with a microliter syringe needle to 10 μl of Skelly B in a small pointed tube (prepared by heat sealing the end of a 3-cm piece of 4-mm-OD glass tubing). The gland was allowed to soak for at least 1 hr and then the entire solution was injected onto a 40-m Carbowax 20 M capillary column by Grob splitless injection (nitrogen carrier gas). The temperature was programed from 80 to 180°C at 10°/min. Baseline separations of the *E* and *Z* isomers allowed for the detection of less than 2% of the minor isomer when a typical quantity of 10–20 ng of pheromone was found.

## RESULTS

*Eden (Bivoltine Predominating).* First- and second-instar larvae were collected from sweet corn tassels on July 2–4, 1983. These represented progeny from the first flight period, which peaked on June 18 at this site as determined

by a black-light trap located nearby. Analyses of 21 female moths resulting from these larvae indicated a *Z* pheromone strain (Figure 1).

There was no July flight detected at this site in 1983, indicating no univoltine population. Second- and third-instar larvae resulting from a flight that peaked on August 2, 1983, were collected on August 23. Pheromone analyses of 12 individuals again indicated a *Z* strain. This was similar to the earlier collections, although a higher percentage of *E* was detected in several individuals (Figure 1).

In 1984, 22 ECB adults emerged from the corn stubble in a field cage during June 8–25. Analyses of five of these females and eight females from field-collected pupae (June 12) showed that they were all of the *Z* strain. Only one female emerged in July, representing a very small univoltine flight in this area, and that female also was a *Z* borer (see Table 1). Pupae collected from sweet corn on August 1 produced females only of the *Z* type.

*Geneva (Bivoltine Predominating).* Overwintered ECB larvae were collected in 1982 sweet corn stalks from May 25–June 4, 1983. Additional stubble from the same planting was placed under a field cage and a major moth emergence occurred on June 15. A black-light trap at the site showed a flight peaking on June 14. Eggs collected from the moths emerging at this time from the corn stubble were reared to adults. All 25 female moths analyzed were found to be of the *E* pheromone strain ($<5\%$ *Z* isomer) (Figure 1).

On July 5, two female moths emerged from the stubble in the field cage. This correlated well with a small flight that occurred there on July 4, 1983, and also in previous years (Eckenrode et al., 1983). Pheromone analyses of these female moths showed that they were both of the *Z* strain (less than 5% *E*) (Figure 1).

ECB resulting from the August flight were collected on August 25, 1983, as second- through fourth-instar larvae. Pheromone analyses of 18 females showed that *E* was the predominating isomer; however, seven moths possessed $>10\%$ *Z*.

In 1984, 51 ECB moths emerged from corn stubble in the field cage in the period June 7–18. Pheromone analysis of the females showed that the females were of the *E* type or were hybrids. Two females emerged from the stubble on June 23 and then a flush of 16 moths was collected during July 4–18. The two females from June 23 and another eight females analyzed from the July flush were found to be of the *Z* strain. One additional female was found to be a hybrid (66% *E*) (see Table 1).

*King Ferry (Bivoltine and Univoltine).* Although a black-light trap was not operated at this location in June, pupae and late-instar larvae (resulting from eggs laid in June) were collected from wheat and sweet corn on July 22, 1983.

Eggs from the July flight, peaking in black-light trap catches on July 16–18, 1983, were collected from sweet corn on July 21. The resulting 21 females were

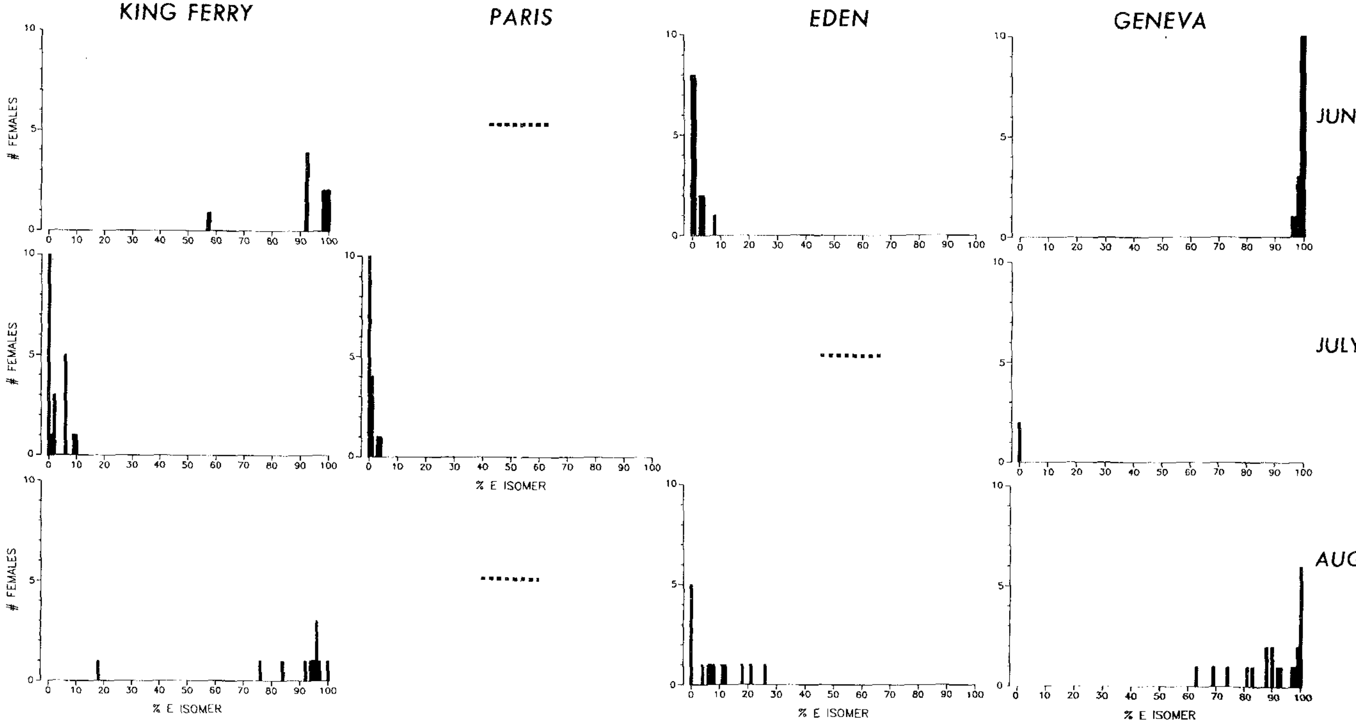


FIG. 1. Single pheromone gland analyses from European corn borer populations collected from selected locations in New York State in 1982 and 1983. Pheromone blend analyses shown for each month are of progeny of that month's flight.

TABLE 1. PHEROMONE ANALYSIS OF FIELD-COLLECTED ECB IN 1984

| | No. of females of each pheromone type | | | | | |
|---|---|---|---|---|---|---|
| | June, bivoltine | | July, univoltine | | August, bivoltine | |
| Locations | *E* | *Z* | *E* | *Z* | *E* | *Z* |
| Eden | 0 | 14 | 0 | 1 | 0 | 16 |
| Paris | No moths | No moths | 0 | 18 | No moths | No moths |
| Geneva | 9[a] | 0 | 0 | 10[b] | 24 | 0 |
| King Ferry | 13[c] | 0 | 0 | 57[d] | not analyzed | |

[a] Also two hybrids (68, 73% *E*).
[b] Also one hybrid (66%, *E*).
[c] Also two hybrids (72, 70% *E*).
[d] Also two hybrids (61, 70% *E*).

found to be of the *Z* strain, with a pheromone of 90% or more *Z* isomer (Figure 1).

Catches in the black-light trap peaked again near King Ferry during the first week of August. Larvae resulting from this third flight were collected as first- through third-instar larvae from sweet corn on August 18, 1983. Analyses of 11 females from these collections (Figure 1) indicated that the *E* isomer was again predominating, although three moths produced less than 90% *E* (84, 76, and 17.5%).

In 1984, 62 moths were collected from the field cage stubble during June 7–29 and 169 moths were collected from the same stubble during July 2–August 3. In the first flight the females were found to contain *E* pheromone or a hybrid blend. In the second flight, all analyzed females were of the *Z* type or hybrid (see Table 1).

Pupae were collected from wheat at Geneva on August 2 and 3 to represent borers of the third flight period. All females analyzed were found to be of the *E* strain.

*Paris (Univoltine).* Black-light trap catches at the Paris site in 1981 (Eckenrode et al., 1983), 1982, and 1983 (unpublished data[2]) showed a typical univoltine pattern with a single flight in July. Overwintering larvae (source of moths for 1983) collected from this site in the fall of 1982 produced 16 adult females that were all of the *Z* strain (less than 5% *E*) (Figure 1).

In 1984, adults did not emerge from corn stubble placed in the field cage on June 7 until June 24. A flush of 65 moths was collected from June 24–July 19, and the analyzed females were found to be of the *Z* type (see Table 1).

*Mixed Populations.* In 1984, larvae were collected from sweet corn at the end of July from three areas located approximately midway between the Geneva location of the bivoltine *E* population and the Eden location of the bivoltine *Z*

population. Moths emerging in August from these collections were analyzed and were found to be 25 *E* females, 7 *Z* females, and 5 hybrids (59–78% *E*).

## DISCUSSION

Data in Figure 1 and Table 1 indicate that at least three distinct ECB populations occur in central and western New York State at the present time, including a bivoltine *Z* population in Eden, bivoltine *E* populations in Geneva and King Ferry, and univoltine *Z* populations in Geneva, King Ferry, and Paris. The field cage studies are particularly revealing for supporting these conclusions because corn stubble from each area was conditioned under the same field conditions at Geneva, and moths emerged in distinct flight periods from the original stubble source. The complete shift from all *E* moths in June to all *Z* moths in July in the Geneva and King Ferry populations shows that these two flights represent distinct populations and that they do not represent differences in host feeding. Ratios of *E*/*Z* isomers varied more in progeny from August flights than progeny from the first flight, possibly indicating that hybridization occurs more readily between moths of the second and third flights. At the present time, the *Z* population near Paris, New York, apparently is isolated from bivoltine types since there is no indication of hybrids, and light trap catches continue to indicate a single flight. Although hybridization and population overlap occurs in many areas of New York (as evidenced by our mixed population analyses), the existence of at least three distinct populations has been documented. Further research with cultures of these populations will be conducted to determine if they can be given race status.

Although Klun and Maini (1979) found hybrids that contained only a 65:35 *E*/*Z* ratio with the populations in their study, our results indicate that blends in New York occur in other *E*/*Z* ratios as well (Figure 1). A similar situation occurred with ECB populations in southern Switzerland where a southern *E* strain from Italy overlapped with a *Z* strain from central Europe (Buechi et al., 1982; Anglade et al., 1984).

Showers et al. (1975) documented the presence of three ECB ecotypes in North America based on diapause response: a univoltine population in Minnesota and Quebec; a bivoltine population in the central states; and a multivoltine population in the southern states. These populations all produced the *Z* pheromone (Klun et al., 1973; Showers et al., 1974; Klun, 1975). The presence of a bivoltine *E* strain was not reported from those regions. The present results provide a basis for improved characterization of varying ECB populations and will aid in clarifying the fluctuation and interaction of the races at different sites.

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# ORIENTATION BEHAVIOR OF SECOND-INSTAR LARVAE OF EASTERN SPRUCE BUDWORM *Choristoneura fumiferana* (Clem.) (LEPIDOPTERA: TORTRICIDAE) IN A Y-TYPE OLFACTOMETER

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**Abstract**—A two-choice, wind-tunnel olfactometer was designed and contructed to determine whether second-instar eastern spruce budworm larvae, *Christoneura fumiferana* (Clem.), could detect and discriminate among host-plant volatiles. Volatiles of current year's growth of *Picea glauca* were preferred over those of *P. rubens*, *P. mariana*, or air. *Abies balsamea* was preferred over *P. mariana* or air. *P. rubens* and *P. mariana* were both preferred over air. Two-year-old growth of *P. glauca*, *A. balsamea*, *P. rubens*, and *P. mariana* were all preferred over air. Current year's growth of these host evergreen species was usually preferred over former year's growth of the same species in each case.

**Key Words**—Eastern spruce budworm, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, conifers, *Picea* sp., *Abies* sp., host volatiles, attraction, larval olfaction, olfactometer.

## INTRODUCTION

The eastern spruce budworm, *Choristoneura fumiferana* Clem. (Lepidoptera: Tortricidae) is a serious pest of spruce/fir forests of Eastern Canada and the United States. Second-instar larvae emerge in early May and feed on developing buds of newly opened shoots of coniferous host trees. Balsam fir (*Abies balsamea*) is usually the most damaged species, followed by white spruce (*Picea glauca*), red spruce (*P. rubens*), and black spruce (*P. mariana*). This order may be related to host tree phenology: balsam fir budburst coincides with emergence of larvae from their hibernacula, four days prior to that of white spruce, and



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about 13 days prior to that of red and black spruce (Swaine and Craighead, 1924).

Feeding preferences of sixth-instar larvae for host-plant extracts and for some pure chemicals have been established (Albert and Jerrett, 1981; Albert et al., 1982; Albert, 1982).

Response to odor by spruce budworm adults has been studied (Thorsteinson, 1960), and the antennae were identified as the site of pheromone receptors (Albert et al., 1970). However, no studies have been done on spruce budworm larval olfaction. Single-cell recordings using host-plant odors have been obtained from other lepidopterous caterpillars (Dethier and Schoonhoven, 1969; Dethier, 1980).

Olfactometers have been used to investigate qualitative and quantitative aspects of insect response to odors. Most consist of wind tunnels in which the insect is exposed to odor-treated airstreams, while some systems rely on diffusion gradients of particular odors in a given environment. They have been used with Orthoptera (Rust and Rierson, 1977), Coleoptera (Anderson and Fisher, 1960; Wood and Bushing, 1963; Mustaparta, 1975), Diptera, (Nettles, 1980; Katsoyannos et al., 1980), Hymenoptera (Ferreira, 1979; Brewer et al., 1983; Vet et al., 1983), Lepidoptera adults (Guerra, 1968; Katsoyanos et al., 1980; Lecomte and Thibout, 1981), and Lepidoptera larvae (Sutherland, 1972; Khattar and Saxena, 1978).

The purpose of the present work was to develop a suitable olfactometer for studying qualitative and quantitative olfactory response behavior of second-instar eastern spruce budworm to its host plants' foliage. We wanted to establish if the early instar of this insect could detect odors, and whether it could discriminate between host-plant foliage volatiles.

## METHODS AND MATERIALS

*Experimental Animals.* Second-instar larvae of *Christoneura fumiferana* (Clem.) were obtained from the Forest Pest Management Institute, Great Lakes Forest Research Center, Sault-Ste-Marie, Ontario, after emergence from diapause. Animals were maintained on gauze for 0–3 days at 4°C, without food or water, and were then fed for 24 hr at 28°C on an artificial diet (McMorran, 1965), before being tested in the olfactometer. Only healthy animals were chosen for the tests. This was crudely assayed by the observation of a characteristic pale color and by the amount of feeding activity or locomotion. Animals were used only once in tests.

*Odor Source.* Foliage samples of white spruce [*Picea glauca* (Moench.) Voss], balsam fir [*Abies balsamea* (L.) Mill], red spruce (*Picea rubens* Sarg.), and black spruce [*Picea mariana* (Mill, B.S.P.)] were collected from the Acadia Forest Experiment Station, New Brunswick, on June 23, 1981, and on June 15,

1982, at a time when larvae were in their fifth and sixth instars in the field. Foliage was also collected on June 6, 1983, when larvae were in their third and fourth instars in the field. All samples were obtained from the mid-crown level of each host. The current year's growth and the previous year's growth were placed in sealed plastic bags over dry ice, then stored at −18°C. Four needles were used as a sample in the bioassay chamber; these were left at room temperature for 5 min prior to the commencement of the assay. Needles were replaced each hour.

*Bioassay.* A diagram of the olfactometer is shown in Figure 1. Airflow was controlled with a tap for coarse adjustment and with a vent clamp for fine adjustment. The air was purified by passing through a charcoal filter (A), a drierite filter (B), a gas purifier including a second drierite filter (C), and a molecular sieve (D), followed by a gas washer (E) containing deionized glass-distilled water. This provided the air with a constant purity, relative humidity, and ambient temperature. Ambient air temperatures varied between 20 and 25°C. The airstream was then divided into two equal parts by a polypropylene Y-type connector and monitored by Roger Gilmont flowmeters (F). Flow rates were controlled by metal clamps on plastic tubing of 0.3 cm diameter. Since the position of the Y-tube was randomly (Sokal and Rohlf, 1969) switched (right and left sides) as an additional control, the airflow was also randomly switched to each branch of the Y-tube. The tube was enclosed in a black plywood box and was illuminated through a light diffusor by a fluorescent light source (G). Air descended into the two branches of the tube at a flow rate of 175 ml/min with a velocity of 614 cm/min (the diameter of the Y-tube was 0.6 cm), after passing through polypropylene joints (H) glued to the tube with epoxy resin. These joints held a layer of gauze (H) which was changed after 1 hr of use. The gauze supported the foliage sample (I). In this manner, the foliage was kept away from the larva. The glass Y-tube (K) held within it a Y-shaped copper wire (L), on which the insects were allowed to crawl. Eight such tubes were constructed and each was tested for bias. All odorous air was carried away in a 0.7-cm Tygon tube to a fume hood in order not to subject future experimental animals to test odors.

To speed up the bioassay, this olfactometer system takes advantage of the fact that larvae are positively phototropic and negatively geotropic.

Insects were handled with a paintbrush in order to minimize injury, and were placed on a straight wire from which they crawled onto the 0.1-cm-diameter wire within the olfactometer. The test began when the insect was on the latter wire, 1 cm from the bottom, and ended when the insect was 1 cm past the junction of the Y. The Y-tube was then inverted, and the insect was allowed to crawl out. The time to complete the test was noted, as was the larva's behavior while performing the test.

Two groups of 10 or 20 values of airstream choices were tested for contingency prior to assimilation into one sample. Values obtained from foliage col-

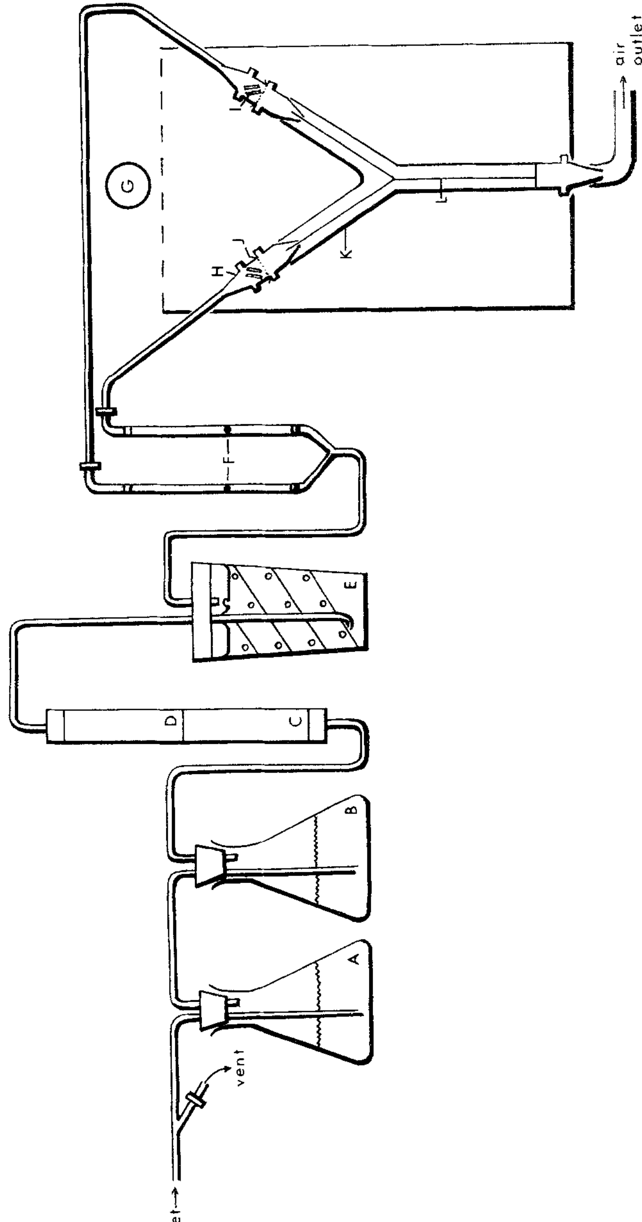


FIG. 1. Schematic diagram of olfactometer. A, charcoal filter, B, drierite filter, C and D, gas purifier, E, gas washer, F, flowmeters, G, light source, H, joint, I, foliage, J, gauze, K, Y tube, L, copper wire.

lected at different times of the year were also tested for contingency. The data were interpreted using a chi-square test with the null hypothesis that the air-stream choice was random. Thus, odor preferences were established. For all chi-square tests, Bate's correction for small sample sizes was used (Sokal and Rohlf, 1969).

The Y-tubes were cleaned after each test, and care was taken to remove all silk produced by the larva.

## RESULTS AND DISCUSSION

*Behavior.* The most common behavior exhibited by the larvae was a persistent crawl, with moderate spiraling around the wire on its way up. This was followed by a rapid decision at the junction of the Y. Once the choice was made, the insect crawled up that branch of the Y more rapidly.

Nine other combinations of exceptional behaviors were exhibited: (1) larvae exhibited klinotaxis (Fraenkel and Gunn, 1961) at 0.5–2.0 cm from the junction of the Y; (2) larvae exhibited klinotaxis at the junction of the Y; (3) larvae crawled down and then back up within 0.75–2.0 cm from the junction of the Y; (4) larvae crawled up the wire in a tight spiral; (5) larvae crawled up one branch of the Y for a few millimeters, then went up the other side (this behavior was usually associated with orientation toward the preferred odor); (6) larvae began to crawl up, and then crawled down, thus not completing the test; (7) larvae crawled all the way up on one side of the wire; (8) larvae crawled very slowly, with many pauses on the way up; (9) larvae exhibited klinotaxis after passing the junction of the Y.

The percentages of the total number of larvae tested which exhibited each of these behavioral sequences are presented in Table 1.

TABLE 1. BEHAVIOR EXHIBITED BY SECOND-INSTAR LARVAE OF *Choristoneura fumiferana* EXPRESSED AS PERCENTAGE OF TOTAL NUMBER OF INSECTS TESTED ($N = 681$)[a]

| Behavior | % of Total insects |
|---|---|
| Most common | 56 |
| Exceptions | |
| 1 | 27 |
| 2 | 18 |
| 3 | 16 |
| 4 | 12 |
| 5 | 12 |
| 6 | 2 |
| 7 | 2 |
| 8 | 2 |
| 9 | 1 |

[a]Refer to results section for description of behavior.

*Choices.* Table 2 outlines the choices made by the second-instar larvae in the olfactometer. Foliage collected in early June yielded the same results as foliage collected later in June.

A significant preference was demonstrated for the host-tree volatiles over air alone. New white spruce is preferred over new red or black spruces; new balsam fir is preferred over new black spruce. New foliage is preferred over the old foliage from the same host.

Table 3 is a compilation of the mean times for at least 40 insects to complete the test and the length of time taken for each choice to be made. There was no significant difference between the times taken to complete the test in the presence of an odor source or in pure air, as determined by confidence limits set using the Student's $t$ test.

TABLE 2. RESULTS OF OLFACTOMETER TESTS WITH SECOND-INSTAR LARVAE OF *Choristoneura fumiferana*[a]

| Choice | | | *f* | | | Chi-square for | | |
|---|---|---|---|---|---|---|---|---|
| I | II | III | I | II | III | I and II | $P^b$ | Preference |
| A | B | X[c] | 53 | 48 | 6 | 0.26 | e | none |
| WSn | BFn | X[c] | 21 | 19 | 2 | 0.12 | e | none |
| WSn | RSn | X[c] | 28 | 12 | 2 | 6.42 | c | I |
| WSn | BSn | X[c] | 28 | 12 | 7 | 6.42 | c | I |
| WSn | air | X[c] | 30 | 10 | 1 | 10.02 | a | I |
| BFn | RSn | X[c] | 24 | 16 | 5 | 1.62 | e | none |
| BFn | BSn | X[c] | 30 | 10 | 2 | 10.02 | a | I |
| BFn | air | X[c] | 32 | 8 | 4 | 14.42 | a | I |
| BFn[d] | air | X[c] | 17 | 3 | 4 | 9.85 | a | I |
| RSn | Bsn | X[c] | 18 | 22 | 2 | 0.50 | e | none |
| RSn | air | X[c] | 31 | 9 | 3 | 12.12 | a | I |
| BSn | air | X[c] | 31 | 9 | 2 | 12.12 | a | I |
| WSo | air | X[c] | 15 | 5 | 2 | 5.05 | c | I |
| BFo[d] | air | X[c] | 17 | 3 | 1 | 9.85 | a | I |
| RSo | air | X[c] | 17 | 3 | 4 | 9.85 | a | I |
| BSo | air | X[c] | 15 | 5 | 2 | 5.05 | c | I |
| WSn | Wso | X[c] | 16 | 4 | 5 | 7.25 | b | I |
| BFn[d] | BFo[d] | X[c] | 16 | 4 | 6 | 7.25 | b | I |
| RSn | RSo | X[c] | 14 | 6 | 3 | 3.25 | e | none |
| BSn | BSo | X[c] | 15 | 5 | 2 | 5.05 | c | I |
| R | L | X[c] | 272 | 308 | 65 | 2.23 | e | none |

[a]BF = balsam fir; BS = black spruce; RS = red spruce; WS = white spruce. n = current year growth; o = two-year-old growth. Chi-square statistics: df = 1.
[b]Probability values: a(<0.005); b(<0.01); c(<0.025); d(<0.05); e(>0.05).
[c]Larvae did not complete the test.
[d]Foliage collected June, 1982; all others from 1981 and 1983 collection.

TABLE 3. MEAN TIME (min) TAKEN FOR COMPLETION OF OLFACTOMETER TESTS BY SECOND-INSTAR *Choristoneura fumiferana* IN PRESENCE OF ODORS OR PURE AIRSTREAM[a]

| Choice | | I | | | II | | |
|---|---|---|---|---|---|---|---|
| I | II | *N* | Mean time (min) | 95% CL | *N* | Mean time (min) | 95% CL |
| Air | air | 53 | 2.12 | 1.60–2.64 | 48 | 1.85 | 1.45–2.25 |
| WSn | air | 30 | 1.67 | 1.28–2.06 | 10 | 1.45 | 1.07–1.82 |
| BFn | air | 32 | 1.79 | 1.43–2.14 | 8 | 2.20 | 1.37–3.02 |
| RSn | air | 31 | 2.02 | 1.62–2.41 | 9 | 2.36 | 1.41–3.31 |
| BSn | air | 31 | 2.10 | 1.75–2.45 | 9 | 2.16 | 1.23–3.08 |

[a]Confidence limits were set using Student's t distribution. WSn = 1983 new white spruce; BFn = 1983 new balsam fir; RSn = 1983 new red spruce; BSn = 1983 new black spruce. 95% CL = 95% confidence limits.

This olfactometer system is an effective one for the second-instar eastern spruce budworm larva. The latter can detect odors since it can consistently move in the direction of an odor stream. It can also dependably choose the same hierarchy of odors as follows: new or old foliage > air; new foliage > old foliage; new white spruce foliage = new balsam fir foliage; new white spruce foliage > new red spruce foliage; new white spruce foliage > new black spruce foliage; new balsam fir foliage = new red spruce foliage; new balsam fir foliage > new black spruce foliage; new red spruce foliage = new black spruce foliage.

It has been postulated (Thorsteinson, 1960) that olfaction serves "to inhibit locomotion away from the plant" in lepidopterous larvae. In the eastern spruce budworm, this would serve as a mechanism for ensuring that larvae hatching from eggs deposited on a host plant previously selected by the adult female would remain on that plant. However, it is worth noting that there was no significant difference in the time taken by larvae to complete the test in the presence or absence of odors. It was also observed that after the junction of the Y, the insect crawled more rapidly. This phenomenon may be due to the resultant decrease in air flow, and/or the fact that the insect is exposed to a single sample. In any case, the insect can orient towards the odors from its host plants, and it can also discriminate between the odors of different hosts. Perhaps at high volatile concentrations the insect's locomotion would be arrested, and the insect would stop to feed, having reached its goal. In the field, the insect can disperse by "ballooning" (Shaw and Little, 1973): suspending itself on a silk thread and being carried by the wind. Although this is most likely triggered by overcrowding, it would be interesting to determine whether an inadequate host could

also provide the necessary stimuli to initiate dispersal. It is conceivable that olfaction could play a role during the period of dispersal if the insect can alight in a suitable area by releasing the silk thread from its spinneret.

Chemical differences exist between the volatiles of spring buds and those of more developed foliage (Von Rudloff, 1972, 1975), but data obtained from using foliage from the period when the insects were in their fifth and sixth instars in the field did not differ significantly from the data obtained using foliage from the period when the insects were in their third and fourth instars.

The antennae of second-instar larvae are quite prominent and must be functional during this stage of the insect's life cycle. The olfactometer used in this study could be used to locate the olfactory sensilla, by performing systematic amputations of antennae and mouthparts. Using this same olfactometer, the chemical components of the host plants could be studied, using odor-treated filter paper disks. The chemical components of related nonhost plants could also be studied to determine whether any deterrent odors are present, which might prevent larvae from settling on the foliage. Such experiments are planned.

Olfaction may play a role in the feeding behavior of spruce budworm larvae. This could be determined by conducting feeding experiments in the presence and in the absence of odors. The odor preferences established in this study correspond closely to the taste preferences found in the work of Albert (1982).

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# INHIBITION OF CYANOGENESIS BY TANNINS

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**Abstract**—During isolation of two biosynthetic types of cyanogenic glycosides from *Carica papaya*, weak cyanide tests were obtained from initial fractions. Upon final purification, strongly positive cyanide tests were obtained. Pretreatment of extracts to remove polyphenolics alleviated inhibition of cyanogenesis, which led us to suspect that tannins were inhibitory agents. Qualitative and quantitative measures of inhibition were made using standard cyanogenic glycosides and polyphenolics. Cyanogenesis was inhibited quantitatively when condensed tannins (quebracho, wattle, and chestnut), or hydrolyzable tannin (tannic acid) were added. When tannins were precipitated from the reaction mixture, cyanide tests proceeded optimally. These results stress the need to interpret negative cyanide tests with care and indicate possible ecological synergisms between plant defensive chemicals.

**Key Words**—Tannins, cyanogenic glycosides, *Carica papaya*, β-glucosidase, tetraphyllin B, prunasin, amygdalin, enzyme inhibition.

## INTRODUCTION

Tannins are widespread in plants, and their ingestion has been shown to have adverse effects on a variety of herbivores. Increased tannin ingestion has been correlated with decreased larval and pupal growth as well as increased larval and pupal mortality in *Operophtera brumata* (Feeny, 1968). These larval lepidopterans showed decreased fitness with addition of 1% condensed tannin to the diet (Feeny, 1968). Some species of acridid nymphs fed greater than 10% dry weight condensed tannin suffered reduced weight and survival as adults (Bernays et al, 1981). Lindroth and Batzli (1984) examined growth of *Microtus ochrogaster* on tannin-enriched diets and found survival and growth rates to be inversely related to tannin concentration.

It has been suggested that tannins function by reduction of foodstuff diges-



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tibility due to their ability to complex with nutritive proteins and digestive enzymes (Feeny, 1970, 1976; Levin, 1976; Rhoades and Cates, 1976; Rhoades, 1979). While it is difficult to separate antifeedant from direct effects, it is true that tannins complex with and precipitate proteins. Details of association and disassociation of these complexes have been described (Mejbaum-Katzenellenbogan et al., 1959). Tannin–protein complexes have been found to be very stable (Van Buren and Robinson, 1969) due to covalent condensations as well as hydrogen bonding (Loomis and Battaile, 1966). More recently the binding specificities of tannin and protein interactions have been measured using a competitive binding assay (Hagerman and Butler, 1981). The observed stability of tannin–protein complexes has been corroborated by structural elucidation of binding sites in protein complexes with proanthocyanidins and gallic acid esters (McManus et al., 1983). Reaction mechanisms have been reviewed by Van Sumere et al. (1975).

Support for the idea of a direct action of tannins on digestive enzymes has been assembled in several in vitro studies. Inhibition of enzyme activity by oak tannins has been shown for trypsin hydrolysis of casein protein (Feeny, 1969) and $\alpha$-amylase hydrolysis of starch (Gadal and Boudet, 1965). An influence of tannins upon enzyme action has been implied for cellulases and pectinases involved in the ripening of fruits in some early studies (Barnell and Barnell, 1945; Hathway and Seakins, 1958). Wattle tannin and tannic acid have been tested for their ability to inhibit enzyme activity in order to elucidate a possible role of tannins in fruit ripening (Goldstein and Swain, 1965). This study demonstrated precipitation of several enzymes by tannins, including a commercially available $\beta$-glucosidase fraction, as measured by loss of enzyme activity.

Cyanogenic glycosides are known toxins (Jones, 1962, 1981) which have been demonstrated to be ecologically important deterrents to herbivory (Angseesing and Angseesing, 1973; Cooper-Driver and Swain, 1976; Bernays et al. 1977; Woodhead and Bernays, 1978). Their deterrency is manifested only in the release of hydrogen cyanide upon enzymatic hydrolysis.

*Carica papaya* L. (Caricaceae) contains the cyanogenic glycosides tetraphyllin B and prunasin (Spencer and Seigler, 1984a). During isolation of these two biosynthetic types of cyanogenic glycoside, only weak cyanide tests were obtained. This phenomenon had previously been noted during isolation of prunasin from *Passiflora edulis* (Spencer and Seigler, 1983). Prunasin is known to be hydrolyzed efficiently by a $\beta$-glucosidase specific for aromatic cyanogenic glycosides (Hosel, 1981). Tetraphyllin B is efficiently hydrolyzed by a $\beta$-glucosidase specific for cyclopentenoid cyanogenic glycosides (Spencer and Seigler, 1982a, 1984b). Upon final purification of the cyanogenic glycoside fractions, rapid hydrolysis took place with addition of appropriate enzyme fractions, and positive cyanide tests were obtained. Pretreatment of the extracts to remove polyphenolics alleviated inhibition of cyanogenesis, which led us to suspect that tannins were inhibitory agents.

The present study was carried out in order to determine the potential for the existence of a synergistic interaction between the protein-precipitating tannins and the enzyme-mediated production of cyanide within tissues of a single plant.

## METHODS AND MATERIALS

*Plant Materials.* Dried leaves of *Carica papaya* L. were purchased commerically (Corn Country Products, Champaign, Illinois). Fresh leaves of *P. quadrangularis* L. were obtained from cultivation at the University of Illinois, Urbana. Voucher specimens are on file at the university herbarium. Fresh leaves of *Polypodium californicum* L. were obtained from cultivation at the University of California, Irvine. Fresh material of *Adenia digitata* Engl. was obtained from Abbey Gardens, Carpenteria, California.

*Isolation of Tannins from Carica.* Dried leaves of *Carica papaya* (35.1 g) were soaked in 80% MeOH homogenized. The mixture was covered and refrigerated for 12 hr. The mixture was then filtered and the solid material was extracted twice with 80% MeOH, filtered, and the procedure repeated using $Me_2CO$ and finally distilled $H_2O$. The filtrate was then concentrated under vacuum to a thick syrup. This was brought up to volume in 100 ml of MeOH, and 10 ml of this solution was then diluted to a total volume of 100 ml and read colorimetrically at 725 nm.

*Isolation of Glycosides.* Prunasin was isolated from leaves of *Polypodium californicum* as previously described (Spencer and Seigler, 1983; Spencer, Seigler, and S. Whitmore, unpublished). Tetraphyllin B was isolated from *Adenia digitata* as previously described (Spencer and Seigler, 1982a,b). Amygdalin was obtained commercially (Calbiochem).

*Qualitative Determination of Cyanide.* Emulsin was obtained commercially (Sigma). Emulsin is a partially purified $\beta$-glucosidase enzyme fraction capable of hydrolyzing aromatic cyanogenic glycosides (Hosel, 1981). *Passiflora quadrangularis* enzyme preparation was made by grinding 100 g of fresh leaves in 500 ml $Me_2CO$ followed by filtration and rinsing with additional $Me_2CO$. The solid in the filter was dried under vacuum conditions and suspended in 500 ml against pH 6.8 buffer. The final product was concentrated under vacuum to 50 ml and its activity tested using fresh plant material according to the Feigl-Anger method. *Passiflora quadrangularis* enzyme preparation efficiently hydrolyzes cyclopentenoid cyanogenic glycosides (Spencer and Seigler, 1984b).

HCN released by $\beta$-glucosidase hydrolysis of cyanogenic glycosides was tested using the Feigl-Anger method (Feigl and Anger, 1966). Strips of Whatmann 3 MM paper were soaked 1–2 min in 1% 4,4-tetramethyldiaminediphenyl methane (tetrabase) in $CHCl_3$ and 1% (w/v) copper ethylacetoacetate in $CHCl_3$ and were dried. The strips turn blue upon exposure to HCN. One milliliter each

of a 1.0 M solution of cyanogenic glycoside and the corresponding enzyme (1 mg/ml for emulsin, a concentration of similar activity of the other preparations) were placed in a vial with Feigl-Anger strip held in place above the liquid with a stopper. Amygdalin with emulsin, prunasin with emulsin, and tetraphyllin B with *P. quadrangularis* enzyme preparation each gave positive cyanide tests as indicated by the Feigl-Anger method. Enzyme specificity for each substrate was shown to be in accordance with previous reports (Spencer and Seigler, 1984b).

*Quantitative Determination of Cyanogens.* Quantitative measurements of cyanide were made using a modified Lambert procedure (Lambert et al., 1975). Standard NaCN solution was prepared as 1.0 ml 0.01 M NaCN in 1.0 M NaOH diluted to 50 ml with 1.0 M NaOH. Trial tubes contained 0–500 $\mu$l NaCN each, then made up to a volume of 1.0 ml with 0.1 M NaOH. Acetic acid, 0.5 ml 1.0 M, was then added to each trial tube. Succinimide–*N*-chlorosuccinimide (5.0 ml), made up in the proportion 2.5 g succinimide to 0.25 g N-chlorosuccinimide per liter of distilled water was then added. Finally 1.0 ml of barbituric acid–pyridine reagent, comprised of 12.0 g barbituric acid and 120 ml pyridine made up to a final volume of 400 ml with distilled water, was added to each tube. Five dilutions each of triplicate samples were vortexed and the optical density read after a 10-min period at 580 nm. A standard curve was then prepared.

Qualitative tests for HCN were carried out using 1.0 ml of 1.0 M solution of the cyanogenic glycoside and 1.0 ml of the appropriate enzyme or enzyme preparation (see above) placed together in the outer well of a Warburg flask while 0.5 ml 0.1 M NaOH was placed in the center well. Flasks were stoppered tightly and incubated at room temperature for 12 hr. The contents of the center well were then removed with a Pasteur pipet and diluted 1:20 with 0.1 M NaOH. Appropriate dilutions were made (as for the standard NaCN solution) and determinations of cyanide made using the modified Lambert method.

*Quantitative Determination of Tannins.* Quebracho tannin from *Schinopsis balansae* Engl. was obtained from the Van Dyke Supply Company, Woonsockett, South Dakota; wattle tannin from *Acacia mearnsii* DeWild and chestnut tannin from *Castanea sativa* L. were the gifts of D.S. Seigler. Tannic acid, quercetin, and rutin were purchased commercially (Sigma).

Total phenolics in the above polyphenol preparations were determined by the Folin-Denis method (Folin and Denis, 1915; Folin and Ciocalteau, 1927; Rhoades, 1977). The Folin-Denis reagent was prepared by adding 41.25 g $Na_2WO_4 \cdot 2H_2O$, 8.25 g $20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$, and 20 ml 85% phosphoric acid to 300 ml distilled water. The mixture was refluxed 2 hr, cooled, and diluted to 1 liter in distilled water. A blank of 1.0 ml 80% MeOH + 1.0 ml Folin-Denis reagent + 1.0 ml $Na_2CO_3$ (1.0 M) was prepared with vigorous shaking upon each addition. Absorbance was measured at 725 nm. Polyphenolic preparations ranging from $10^{-7}$ g/ml MeOH to 0.10 g/ml in 80% MeOH were measured at 725 nm.

*Inhibition of Hydrolysis of Cyanogenic Glycosides by Tannins.* Tannins were added to Warburg flasks containing the three cyanogen sources and corresponding $\beta$-glucosidase fractions. Six flasks were used for each run, the first containing no tannin and the rest making up a range of tannin concentration from 0.01 mg/ml to 100mg/ml. Tannin (1.0 ml), 1.0 ml of cyanogenic glycoside (474 mg amygdalin; 295 mg prusasin; 287 mg tetraphyllin B), and 1.0 ml enzyme preparation were placed in the outer well, with 0.1 M NaOH (0.5 ml) placed in the center well. Amounts of active $\beta$-glucosidase added were not precisely measured, but each enzyme preparation was adjusted in volume so that 1.0 ml completely hydrolyzed 1.0 ml of a 1.0 M solution of substrate in 12 hr. After 12 hr, the contents of the center wells were removed and treated as previously described. Tests were also carried out after tannins were precipitated with a saturated caffeine solution and with a 1.0% gelatin solution (Segleman and Farnsworth, 1969). The mixture was centrifuged and additional enzyme added. A final assay for cyanide was made using the Feigl-Anger method.

## RESULTS AND DISCUSSION

Feigl-Anger cyanide tests (Feigl and Anger, 1966) were positive for all standard enzyme–cyanogenic glycoside mixtures. In the presence of excess quebracho, wattle, or chestnut tannin as well as tannic acid, results of all Feigl-Anger tests were negative. Extracts of *Carica papaya*, which contains tetraphyllin B and prunasin (Spencer and Seigler, 1984a), also failed to yield cyanide upon treatment with enzyme fractions known to hydrolyze these cyanogenic glycosides. In the presence of the flavonoids quercetin or rutin, all Feigl-Anger tests were positive. Upon precipitation of tannins by casein, caffeine, or gelatin, addition of enzyme to both standard and samples of treated extract gave positive tests for cyanogenesis. These results indicate that the presence of tannins inhibited enzymatic hydrolysis of cyanogens, while the presence of flavonoids did not.

The degree of inhibition of cyanogenesis in vitro was tested quantitatively by varying the amount of tannin added to each cyanogenic glycoside–enzyme mixture and subsequently measuring the amount of cyanide released after 12 hr.

Figure 1 shows the degree of inhibition of hydrolysis of amygdalin by emulsin in the presence of tannin. Wattle, chestnut, and quebracho tannins and tannic acid all inhibited hydrolysis. Quebracho tannin was found to be twice as active as either wattle or chestnut tannin. Tannic acid was found to be approximately three times as active an inhibitory agent as was the same concentration of any of the condensed tannins. Significant inhibition occurred upon addition of tannins in concentrations representing 0.03–3.0% of the reaction mixture in all cases. Halving the amount of amygdalin present in the reaction mixtures resulted in a decreased inhibitory effect of the tannins. Analysis of these diluted mixtures yielded straight line plots reflecting incompletion of hydrolysis.

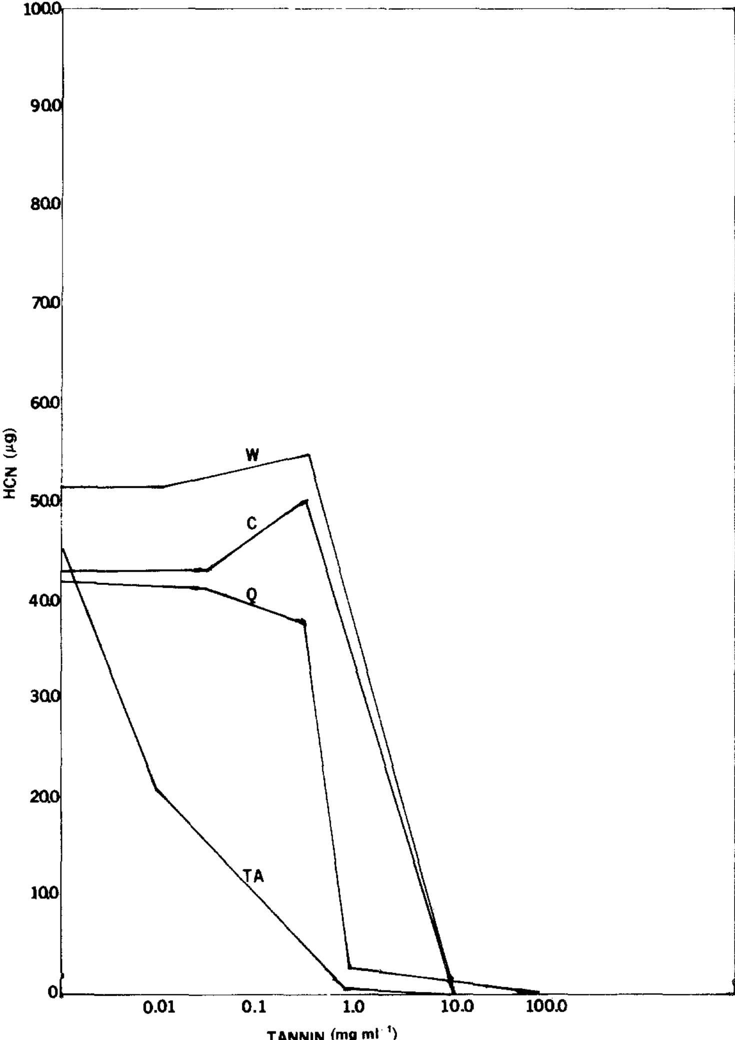


FIG. 1. Inhibition of hydrolysis of amygdalin by emulsin upon addition of tannin. TA = tannic acid; Q = quebracho tannin; C = chestnut tannin; W = wattle tannin.

Figure 2 represents the degree of inhibition of hydrolysis of prunasin by emulsin in the presence of tannins. Wattle, chestnut, and quebracho tannins all inhibited hydrolysis. Chestnut tannin was found to be approximately 1.5 times as active an inhibitory agent as wattle tannin. The threshold of inhibition occurred with 1.0 mg/ml tannin, below which point the lower concentrations of tannin no longer significantly inhibit cyanogenesis, as was found for amygdalin.

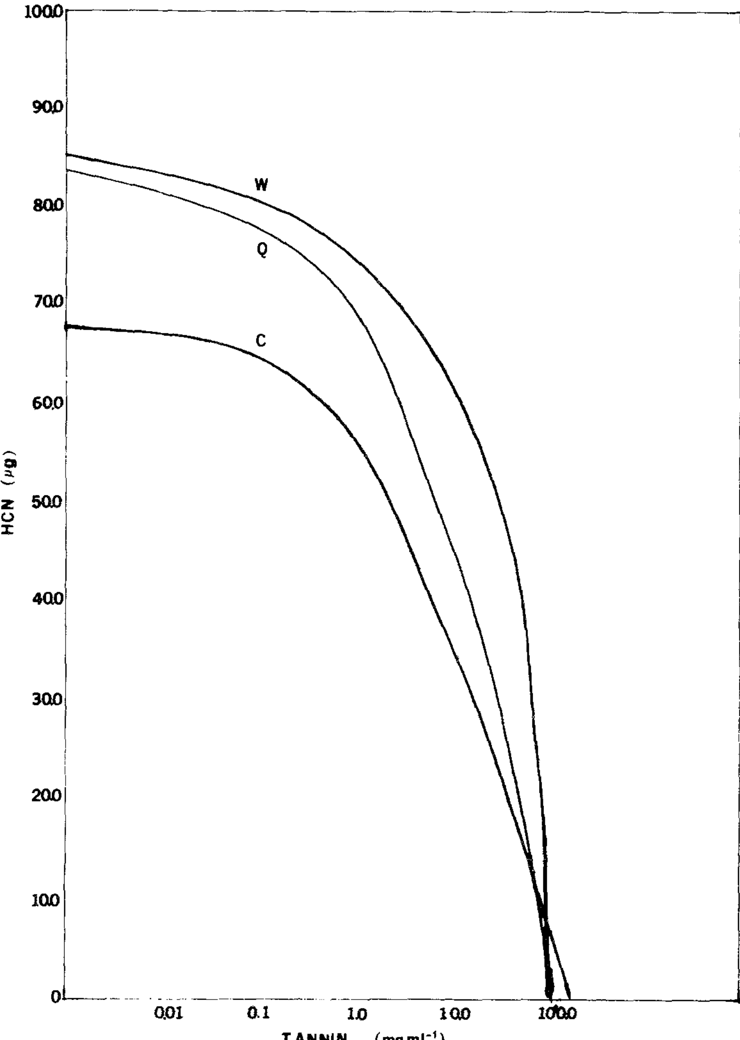


FIG. 2. Inhibition of hydrolysis of prunasin by emulsin upon addition of tannin. W = wattle tannin; Q = quebracho tannin; C = chestnut tannin.

Significant inhibition again occurred at tannin concentrations representing 0.3–3.0% of the reaction mixture.

Wattle, chestnut, and quebracho tannins all inhibited hydrolysis of tetraphyllin B by *P. quadrangularis* enzyme preparation (Figure 3). Thresholds of inhibition occurred at 1.0 mg/ml tannin added to the reaction mixture. Complete inhibition occurred at tannin concentrations above 3.0% of the reaction mixture.

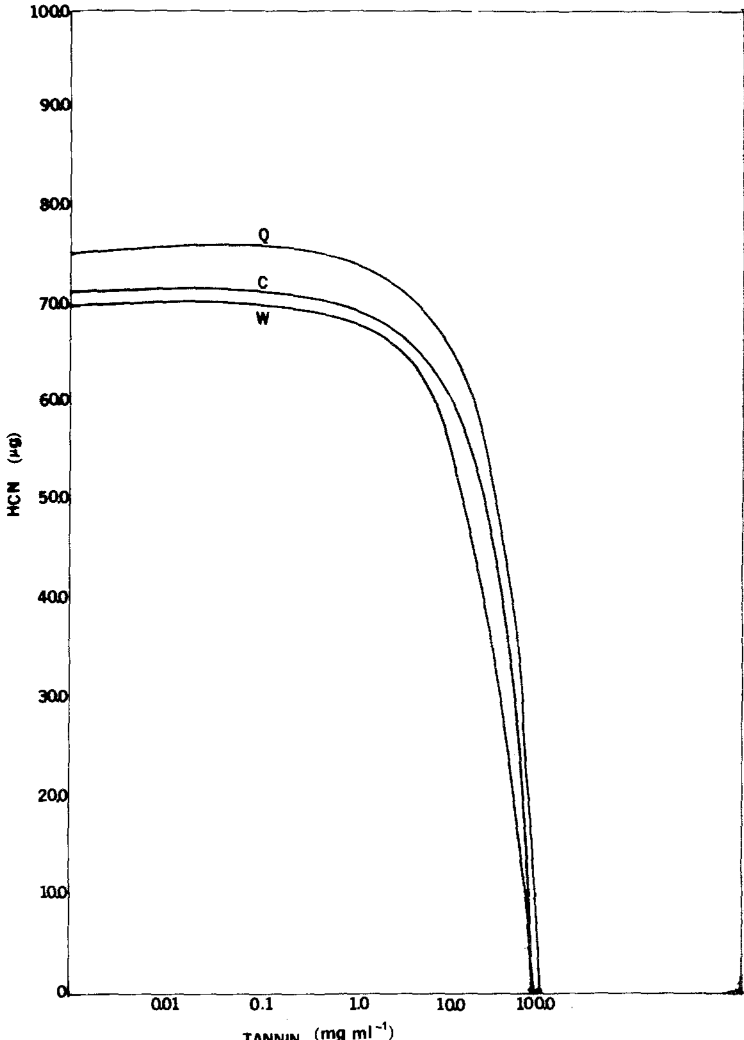


FIG. 3. Inhibition of hydrolysis of tetraphyllin B by a *Passiflora quadrangularis* enzyme preparation. Q = quebracho tannin; C = chestnut tannin; W = wattle tannin.

Figures 1–3 all show inhibition of cyanogenesis by added tannins. A similar threshold response was found in all reactions, except that of the emulsin hydrolysis of amygdalin in the presence of tannic acid where some degree of inhibition was found at all levels. The three condensed tannins were found to show differential inhibitory activity against a given enzyme preparation. No one condensed tannin was found to have the greatest inhibitory effect on all cyanogenic glycosides. Tannic acid, however, was found to have a greater inhibitory effect

than any of the other tannins. This indicates that the structural form and type of tannin used in ecological experimentation may affect the results obtained.

A quantitative measurement of the total phenolics present in leaf extracts of *Carica papaya* was made using the Folin-Denis (Folin and Denis, 1915; Folin and Ciocalteau, 1927; Rhoades, 1977) technique. Standard absorbance–concentration curves were prepared for all of the tannins used in this study (Figure 4). Comparison of total phenolics in *C. papaya* with these standard curves shows our samples to contain approximately 2.3% dry weight polyphenolics. This re-

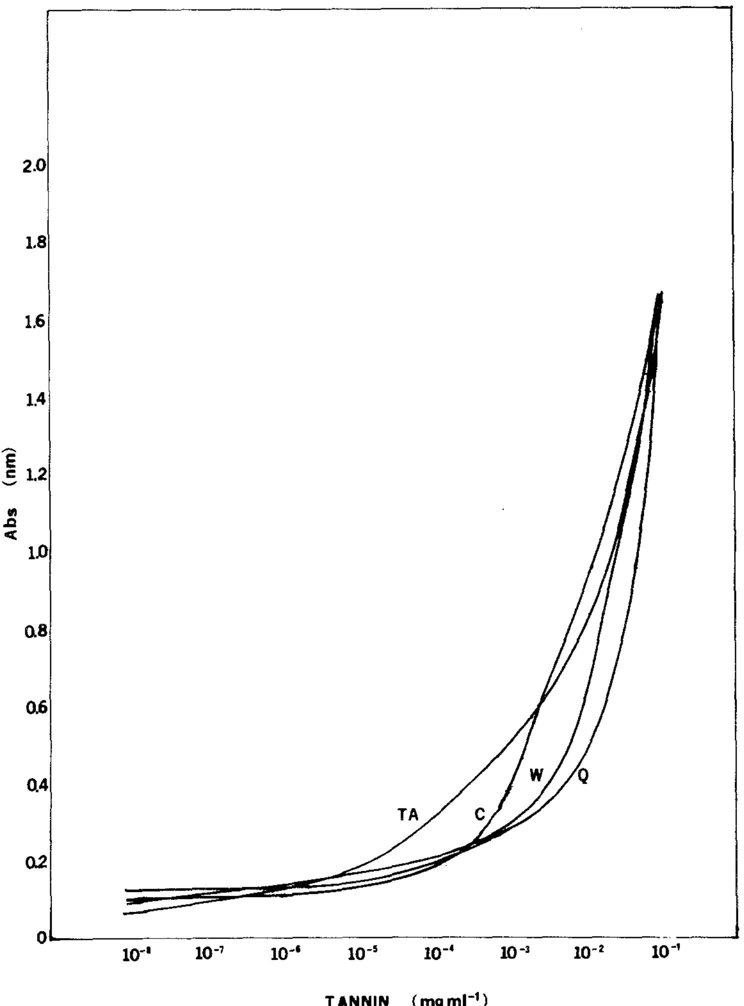


FIG. 4. Standard absorbance curves prepared by the Folin-Denis colorimetric method. TA = tannic acid; C = chestnut tannin; W = wattle tannin; Q = quebracho tannin.

sult is in a similar range to those of Feeny (1970) and Feeny and Bostock (1968), who found 0.5–5.0% dry weight polyphenolics in oak leaves (*Quercus robur* L.). We also measured polyphenolics in *Quercus agrifolia* Nee and found them to be present as 2.17% dry weight. The cyanogenic glycosides of *C. papaya* have been isolated as 0.019% dry weight (Spencer and Seigler, 1984), indicating that sufficient tannin is present to inhibit cyanogenesis if the tannin and enzyme were allowed to react. It was also found that addition of polyphenolics extracted from *C. papaya* in fact inhibited cyanogenesis when added to prunasin–emulsin and tetraphyllin B–glucosidase mixtures in the same manner as did the tannins from other sources.

Alternative models to the digestibility reduction theory of tannin function have been proposed, including direct toxicity (Bernays, 1980). It has been found that, although tannins have the potential for reducing digestibility of proteins in foodstuffs, the detergency of insect gut fluid may interfere with tannin precipitation of dietary proteins (Martin and Martin, 1984). Studies of the actual effects of phenolics upon digestion have not shown a clear function (Williams, 1959; Jung and Fahey, 1983). Regardless of their mechanism of action, we feel it important to consider their interaction with other secondary metabolic systems.

It is not clear how tannins and cyanogenic glycosides contained in the same plant would interact in natural situations of herbivory. It would, of course, not be expected that β-glucosidases would be precipitated by tannins in normal plant metabolism. These enzymes are probably isolated compartmentally within plant tissues (Kojima et al., 1979).

It is possible that herbivores which are deterred by cyanogenic glycosides do not ordinarily encounter significant amounts of tannins during feeding. Conversely, those herbivores that are not deterred may feed to such a manner that contact between tannin and the glucosidases responsible for toxification is maximized. An inverse relationship between tannin and cyanide content in *Lotus corniculatus* has recently been described (Ross and Jones, 1983).

Our data show that negative test results for cyanogenic glycosides can be caused by the concurrent pressence of tannins. We have shown that tannins inhibit quantitatively the β-glucosidase-mediated hydrolysis of cyanogenic glycosides in vitro. We suggest that in certain ecological situations tannins may interfere with the release of HCN from the hydrolysis of cyanogenic glycosides and thereby reduce their toxic effects.

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# STICKY SECRETION FROM TWO PAIRS OF DEFENSIVE GLANDS OF ROVE BEETLE *Deleaster dichrous* (GRAV.) (COLEOPTERA: STAPHYLINIDAE)

## Gland Morphology, Chemical Constituents, Defensive Functions, and Chemotaxonomy

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**Abstract**—The phylogenetically primitive rove beetle *Deleaster dichrous* (Grav.) (Oxytelinae) has been shown for the first time to possess two pairs of neighboring abdominal glands which are depleted simultaneously on molestation. The morphology of these glands is described. The defensive constituents of the *Deleaster* glands were elucidated directly from the mixtures by gas chromatographic–mass spectroscopic methods and microchemical reactions. The paired whitish glands secrete iridodial, which polymerizes on exposure to air to form an adhesive that probably deters small predatory arthropods. The red gland system of *D. dichrous* contains the toxic *p*-toluquinone and a variety of isopropyl and *sec*-butyl esters. Artificial quinoid ester mixtures simulating the secretion of *D. dichrous* showed only weak effects on mortality of *Lucilia* larvae in comparison with more effective secretions of phylogenetically derived Oxytelinae. The secretion of the primitive genus *Deleaster* is characterized chemotaxonomically by $\beta$, $\gamma$-unsaturated $C_{12}$ acids and esters, which are postulated as precursors for the characteristic defensive compounds of the derived species, thus indicating a clear evolutionary trend at the micromolecular level.

**Key Words**—Coleoptera; Staphylinidae, *Deleaster dichrous*, defensive secretion, gland system, iridodial adhesive, isopropyl esters, *sec*-butyl esters.

## INTRODUCTION

Among Coleoptera, rove beetles are characterized by a lengthened, slender body and reduced elytra covering only parts of the abdomen. The increased abdom-



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inal mobility, which is advantageous for movement within the interstices of any substrate, is of great disadvantage when the staphylinid, with its unprotected abdomen, has to defended itself against any predatory organism. Therefore these beetles have evolved different types of defensive glands situated within the unprotected abdominal region (Araujo, 1978). It seems probable that these glands have evolved recently and rapidly since nearly every rove beetle subfamily is characterized by its own defensive gland system (Araujo, 1978). This morphological diversity of the defensive glands is reflected in the chemistry of secretions, indicating that rove beetles are excellent terpenoid chemists among Coleoptera (Weatherston and Percy, 1978). Thus these beetles provide an excellent basis for studying the evolution of chemical defensive systems and assessing their value in taxonomy.

The subfamily Oxytelinae was chosen as a basis for detailed analysis of the morphology and chemistry of the defensive glands. Interestingly, the abdominal glands associated with the ninth tergite form one of the most important characters uniting all 1700 representatives of this subfamily (Herman, 1970; Newton, 1982). According to these authors, there is an urgent need for comparing morphological and chemical trends of the defensive glands between primitive and highly evolved species of the subfamily. *Deleaster*, which is undoubtedly the most primitive genus within the Oxytelinae, was chosen for this study in order to discover and to define possible trends in its chemical defensive system, as compared with those of phylogenetically derived species.

During several years, only a few specimens of *Deleaster dichrous* were available for this study each month. Therefore the identification of its defensive constituents was possible only by analysis of the complex mixtures using micromethods. Isolation of constituents was not possible because of the scarcity of the beetle material. This paper details the exceptional morphology and chemistry of the abdominal defensive glands of *Deleaster dichrous*. Biological observation such as discharge of the gland reservoirs and repellency tests of artificially simulated secretions against dipteran larvae are also described. Finally, biogenetic and chemotaxonomic questions are discussed.

## METHODS AND MATERIALS

Between 300 and 400 specimens of *Deleaster dichrous* were collected from under rocks near the edge of the river Kall which is situated in the northern Eifel region (Rhineland). Carefully transported beetles were immediately frozen on reaching the laboratory. Excised abdominal glands were macerated with KOH and vitally stained with toluidine blue for morphological studies or kept untreated for chemical work.

For chemical analysis, gland reservoirs were excised from frozen beetles on an ice bath and deposited in the tiny groove of the cooled movable wire

plunger of a 0.1-μl mini-injector (Precision Sampling Corporation). This method allowed injection with or without split and analysis of gland reservoirs of single beetles by capillary gas chromatorgraphy without using any solvent. Gas chromatographic analyses were performed with a Carlo Erba Fractovap 2900 capillary gas chromatograph equipped with a FID detector (carrier gas: helium; 1 ml/min) and a Spectra Physics computer integrator (system I). The following glass capillary columns and temperature programs were used: 8-m CW 20 M (65°C: 2 min isothermal; 65°–225°C: 5°C/min); 20-m WG 11 (65°–225°C: 5°C/min).

Capillary gas chromatography–mass spectrometry (GC-MS) was performed on a Varian 3700 capillary gas chromatograph coupled to a MAT 44 quadrupole mass spectrometer which operated at 80 eV and was connected to a Varian SS 200 computer system. For GC-MS, the following glass capillary columns and temperature programs were used: 8-m CW 20 M (60°C–220°C: 10°C/min); 20-m SE 30 (60°C: 3 min isothermal, 60°–240°C: 12°C/min). Chemical ionization (CI)–mass spectroscopic data were obtained with the same GC-MS system using isobutane as reactant gas (130 eV). For high-resolution (HR) measurements, several glands were deposited into a small aluminum crucible with a cover, which was introduced in a double-focusing mass spectrometer (Varian MAT 311 A). Elemental compositions of molecular masses and fragments of main constituents were determined by using the peak matching method against perfluorokerosine reference samples.

Microscale bromination of unsaturated glandular components prior to GC was performed by adding 2 μl of a 1 : 1 (v/v) solution of bromine in tetrachloromethane to several crushed gland reservoirs deposited in a micro glass tube which was then closed and heated at 150°C (30 min). Similar physical conditions were used for saponification of esters with concentrated aqueous solution of potassium hydroxide or reduction of carbonyl compounds (with saturated ethanolic sodium borohydride) present in the defensive secretions. Formation of trimethylsilyl derivatives of free fatty acids in the defensive secretion was performed by using MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoracetamide). For acylation of the iridodials, TFAA (trifluoroacetic anhydride) was used.

The detection of free fatty acids in the secretion of gland 1 was not possible by esterification with hydrochloric acid–methanol or with boron trifluoride–methanol because of transesterification of the natural isopropyl and *sec*-butyl esters. Therefore, several gland reservoirs were mixed with 5 μl of diethyl ether in a micro glass tube. After centrifugation, the upper phase was sucked off and put into another micro glass tube which was placed (instead of diethyl ether) into the cooled larger tube of a commercial microdiazomethane apparatus (Aldrich). Diazomethane was generated from 100 mg MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine), and 0.5 ml 5 M aqueous potassium hydroxide solution was then used to convert any naturally free acids into their methyl esters.

Authentic methyl, isopropyl, or *sec*-butyl esters of the straight chain and unsaturated esters were prepared from free acids by esterification using the corresponding alcohol and concentrated hydrochloric acid.

Different saturated quinoid solutions (*p*-toluquinone) were prepared according to the main constituents of different oxyteline species. Last-stage *Lucilia* larvae were treated with 1 $\mu$l solution per specimen. Dead larvae were counted two hours and four days after they had been topically treated with artificial quinoid test solution.

Single and subsequent molestations of *Deleaster* specimens were performed by carefully touching the thorax of a beetle with a forceps. Simultaneously the beetle was induced to push its abdominal tip towards a minute filter paper triangle. If this was not successful or if the beetle additionally touched the forceps, the filter paper triangle was rejected and a new beetle was used. By addition of a trace of water, the minute filter paper triangle with centrally adhering defensive secretion was inserted into the groove of the aforementioned wire plunger and was injected in the gas chromatograph.

## RESULTS

### *Morphology of Glands*

When molested by arthropods or conspecifics, the rove beetle *Deleaster dichrous* (Grav.) bends its abdomen dorsally as shown in Figures 1 and 2. Continuous or strong irritations such as biting result in increasing flexion of the abdominal tip and emission of small droplets of a reddish secretion at the attacking animal. Dissections and carefully performed examinations of abdominal tips showed that there are four unique complex gland systems present in *Deleaster dichrous* (Grav.) (Figure 3). Two glands which have been found to be present in all members of the subfamily Oxytelinae are named glands 1 or red glands in view of the reddish gland secretion sequestered within reservoir 1. The other gland system, which is now described for the first time, has been called gland 2 or white gland due to the whitish gland material of reservoir 2. The histology and morphology of both complex gland systems are described below.

*Gland System 1 (Red Gland).* This paired gland system consists of a lengthened gland tube (Figures 3 and 4; gl 1), an efferent duct (ed), and a gland reservoir (res 1). The lightly muscled gland reservoir 1 opens (op) at the anterior border of the subdivided ninth tergite. Apart from the gland chemistry, the association of gland system 1 with the ninth tergite morphologically supports the homology of the *Deleaster* gland system 1 with the known defensive glands of all Oxytelinae beetles investigated (Araujo, 1973; Happ and Happ, 1973; Dettner and Schwinger, 1982; Dettner, 1984). As compared with the body volume,

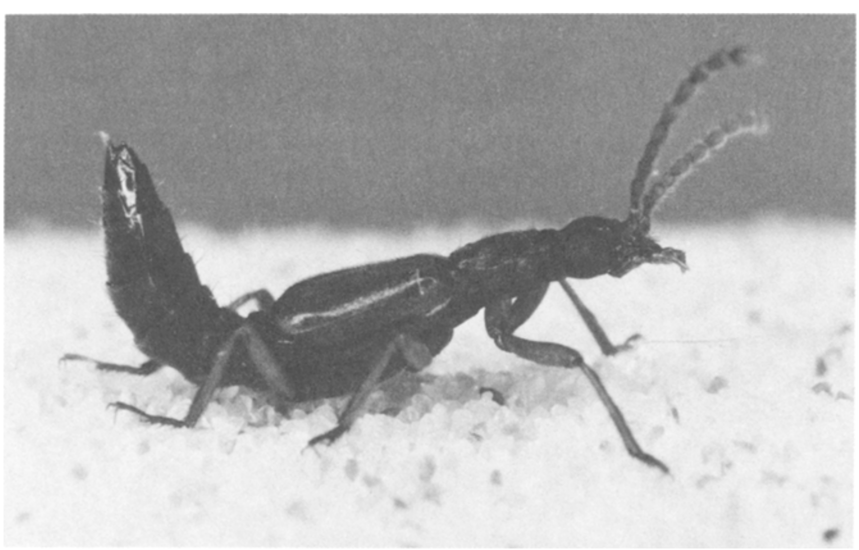

FIG. 1. *Deleaster dichrous* (Grav.) showing its elevated abdominal tip responding to slight irritation. The body length of a beetle ranges from 6.5 to 8 mm.

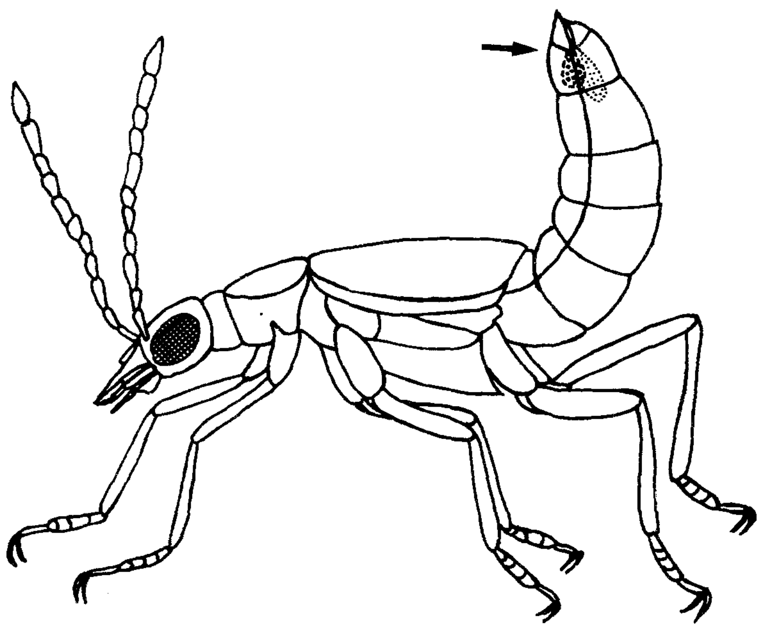

FIG. 2. *Deleaster dichrous* (Grav.) showing its elevated abdominal tip. Arrow occasionally indicates one opening and one reservoir of a red (gland system 1) and a white (gland system 2) gland system.

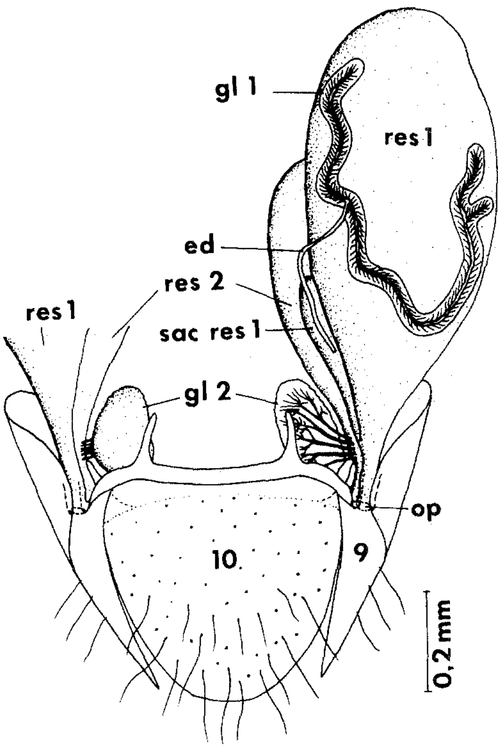


FIG. 3. Dorsal view of an abdominal tip of *Deleaster dichrous* (Grav.) (gl 1, 2: secretory tissue of the gland system 1 and 2, respectively; ed: efferent duct; res: reservoir; sac res: saclike reservoir evagination; op: opening; numbers 1 and 2 indicate gland systems, numbers 9 and 10 indicate tergites). Within the right secretory tissues (gl 1, gl 2) cuticular ductules are outlined.

gland reservoir 1 is small (approximately 1/146 part of the body volume) and shows a characteristic saclike evagination (sac res 1; Figures 3 and 4) originating close to the opening of reservoir 1. Reddish defensive secretion is produced within the lengthened (length at least 1 mm) and apically sometimes branched, gland tube (gl 1), which also shows a lengthened cuticular central cavity (Figure 3; Figure 5A, B, ccc); 300–400 cuticular ductules (cd; Figure 5 B) originate from the cuticular central cavity and lead to the secretory cells. Distally each cuticular ductule loops back and forth about 10 times (s: switchback; Figure 5B, C); its apical region is divided into a 6-$\mu$m long bulb (b, Figure 5B, C) and a 7-$\mu$m long tip (t, Figure 5B, C), which is not extremely lengthened as in derived species of the subfamily (Dettner, Wunderle and Schwinger, in preparation). In other Oxytelinae beetles (Happ and Happ, 1973; Araujo, 1973) the switchback and bulb lie within the cavity of a medullary cell. The tip of a

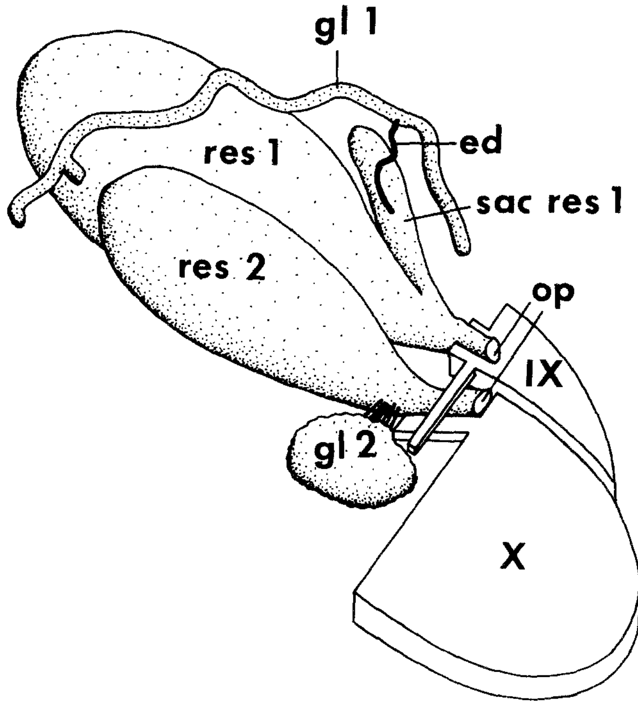


FIG. 4. Semischematic view of one half of *Deleaster* tergites IX and X together with associated defensive gland systems 1 and 2 (unpaired figured); for abbreviations see Figure 3.

ductule projects into a distally situated cortical cell. From the gland tube (gl 1), defensive secretion flows through the shortened 0.6-mm-long efferent duct (ed, Figures 3 and 4) into the sac like evagination (sac res 1) of the gland reservoir 1.

*Gland System 2 (White Gland).* The paired gland system 2 consists of a gland reservoir (res 2; Figures 3, 4, and 5D, E) and an ovoid mass of secretory cells (gl 2; Figures 3, 4 and 5E). Morphologically, gland reservoir 2 is associated laterally with the anterior border of the undivided 10th tergite. Along its longitudinal axis, gland reservoir 1 adheres to gland reservoir 2. Approximately 30 cuticular ductules of secretory cells form a bundle (Figure 3). Near an anteriorly projecting cuticular process of the 10th tergite 15 bundles of cuticular ductules flow into reservoir 2 (Figures 3, 4, and 5D, E). As in gland system 1, each cuticular ductule of the secretory cells of the white gland (Figure 5F) shows a switchback region (s), a bulb (b), and a 3-$\mu$m long tip (t). Therefore the presence of a medullary and cortical cell also has to be inferred for the new described gland system 2.

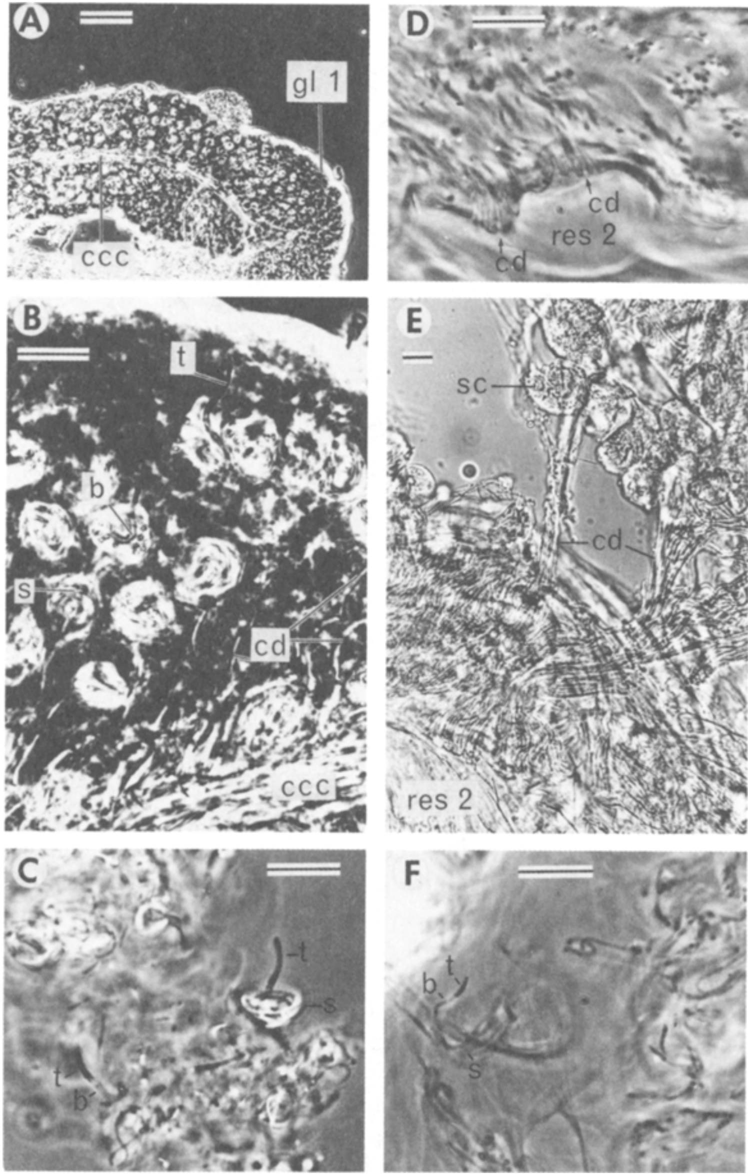


FIG. 5. Defensive gland systems 1 (A–C) and 2 (D–F) of *D. dichrous* (A) Gland tube 1 (gl 1) with cuticular central cavity (ccc). (B) Detail of A showing the cuticular central cavity (ccc), cuticular ductules (cd), switchback region (s), the bulb (b), and tubules (t). (C) KOH macerated gland tube-tissue of the gland system 1. (D, E) Bundles of cuticular ductules (cd) of the gland system 2 leading to gland reservoir 2 (res 2). (F) Detail of the KOH macerated secretory tissue (sc) of E. (abbreviations see Figure 5C). Reference bars: A, E = 50 μm; B–D, F = 10 μm.

*Chemistry of Gland System 1 (Red Gland)*

Defensive molecules of gland system 1 can be subdivided into a volatile fraction and a nonvolatile fraction.

*Volatile Defensive Secretion of Gland System 1.* Seventeen compounds (*1-17*) of the volatile defensive secretion of gland system 1 of *D. dichrous* could be identified (Figures 6 and 7). They represent between 81% and 91%

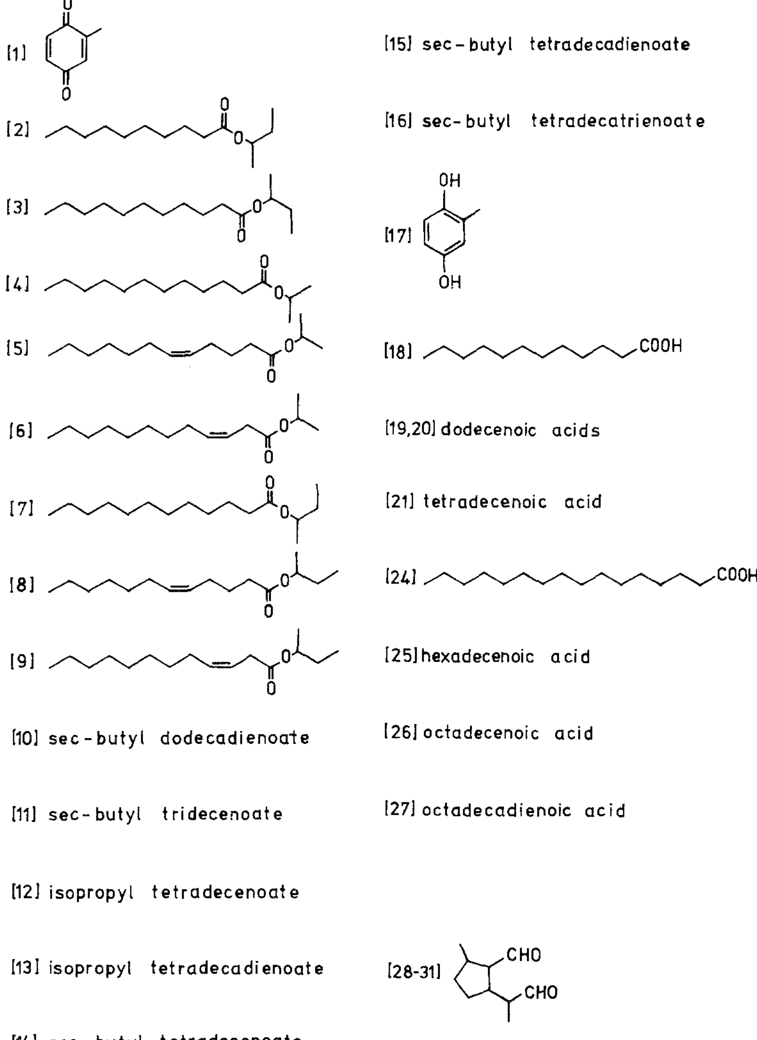


FIG. 6. Volatile and nonvolatile defensive components 1–27 from the red defensive glands (gland system 1) and the whitish glands (components 28–31; gland system 2) of *Deleaster dichrous* (Grav.)

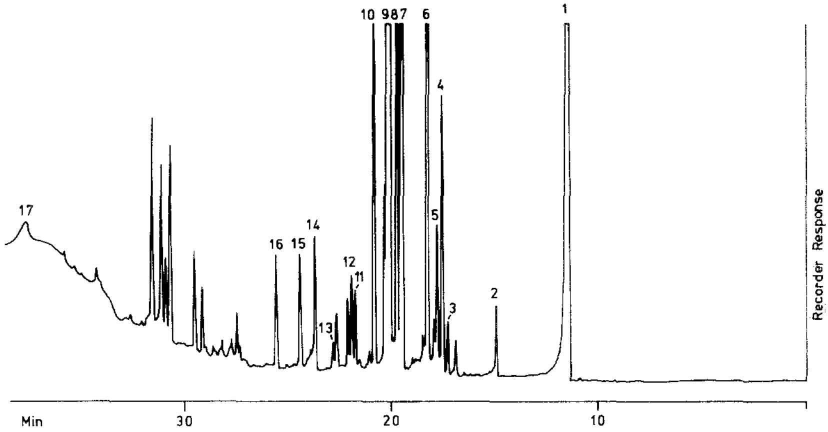


FIG. 7. Capillary gas chromatogram of two defensive gland reservoirs 1 (red glands) from *Deleaster dichrous* by using a 8-m CW 20 M glass capillary. 1: *p*-toluquinone; 17: *p*-toluhydroquinone; 4–6, 12, 13: isopropyl esters; 2, 3, 7–11, 14–16: *sec*-butyl esters (for further explanation see Table 1).

(86.6% ± 3.7%; $N = 8$) of the total peak area found in the gas chromatographic analysis of the red gland secretion of the beetle (Table 1).

*p-Toluquinone (1)*. *p*-Toluquinone represents the main constituent and toxic principle of the red gland secretion of *D. dichrous* and has been found within all Oxytelinae defensive glands investigated (Wheeler et al., 1972; Dettner and Schwinger, 1982; Dettner, 1984). Peak 1 (Figure 7) showed a molecular mass of 122 (CI-MS: 123) and high-resolution measurements of this peak within the crude secretion were in favor of the formula $C_7H_6O_2$ (measured: 122.0373; calculated: 122.03678). Peak 1 cochromatographed with authentic 2-methyl-1,4-benzoquinone, and both samples showed indistinguishable EI mass spectral data; 2.65–3.2 $\mu$g *p*-toluquinone are sequestered per *D. dichrous* individual. As compared with compounds 2–16, the FID detector of the capillary gas chromatograph is characterized by a much lower sensitivity for this quinone (Table 1).

*p-Toluhydroquinone (17)*. The EI mass spectrum of compound 17 revealed a $M^+$ peak at *m/z* 124 (CI-MS: 125) and distinct further fragments at *m/z* 123, 107, 95, 77, 67, 55, and 41. The fragmentation pattern is identical with recorded mass spectral data of authentic *p*-toluhydroquinone (Dettner and Schwinger, 1982). As expected, compound 17 was removed from the gas chromatographic profile of the crude gland secretion on treatment with a silylation agent (MSTFA).

*Saturated Esters (2–4, 7)*. EI mass spectra of compounds 2, 3, 4, and 7 (Figure 7) showed molecular masses of 228 (2), 242 (3, 4) and 256 (7) which were supported by quasimolecular ions (CI-MS) at *m/z* 229 (M + 1; 2), 243 (M + 1; 4) and 257 (M + 1; 7). Characteristically, the molecular ion of 4 loses

TABLE 1. COMPOSITION OF DEFENSIVE GLAND SYSTEM 1 (RED GLAND) OF *Deleaster dichrous* AND ANALYTICAL EVIDENCE FOR ASSIGNMENTS[a]

| Number | Molecular Mass | Compound | Mean % Peak Area Per Beetle (±SD, $N$ = 8) | Range of Values Found (% Peak Area; $N$ = 8) | Range of Values Found (μg/ Beetle; $N$ = 5) | Evidence |
|---|---|---|---|---|---|---|
| 1 | 122 | *p*-toluquinone | 33.16 ± 11.97 | 15.63–49.21 | 2.65–3.2 | GC*,MS:EI,CI,HR,Br+,KOH– |
| 2 | 228 | *sec*-butyl decanoate | 0.20 ± 0.15 | 0.07– 0.45 | 0.01–0.04 | GC*,MS:EI,CI, Br–,KOH+ |
| 3 | 242 | *sec*-butyl undecanoate | 0.14 ± 0.05 | 0.08– 0.22 | 0.01 | GC*,MS:EI, Br–,KOH+ |
| 4 | 242 | *sec*-propyl dodecanoate | 1,34 ± 0.72 | 0.55– 2.83 | 0.02 | GC*,MS:EI,CI, Br–,KOH+ |
| 5 | 240 | *sec*-propyl (*Z*)-5-dodecenoate | 0.45 ± 0.20 | 0.18– 0.76 | 0.01–0.04 | GC ,MS:EI, Br+,KOH+ |
| 6 | 240 | *sec*-propyl (*Z*)-3-dodecenoate | 6.17 ± 2.37 | 2.85–10.45 | 0.08–0.09 | GC*,MS:EI,CI,HR,Br+,KOH+ |
| 7 | 256 | *sec*-butyl dodecanoate | 3.96 ± 1.55 | 2.43– 6.26 | 0.04–0.06 | GC*,MS:EI,CI, Br–,KOH+ |
| 8 | 254 | *sec*-butyl (*Z*)-5-dodecenoate | 1.40 ± 0.89 | 0.42– 2.81 | 0.02–0.03 | GC ,MS:EI,CI, Br+,KOH+ |
| 9 | 254 | *sec*-butyl (*Z*)-3-dodecenoate | 33.83 ± 11.69 | 16.71–48.37 | 0.35–0.37 | GC*,MS:EI,CI,HR,Br+,KOH+ |
| 10 | 252 | *sec*-butyl dodecadienoate | 1.18 ± 0.47 | 0.75– 2.00 | 0.04 | GC ,MS:EI,CI, Br+,KOH+ |
| 11 | 268 | *sec*-butyl tridecenoate | 0.18 ± 0.10 | 0– 0.34 | 0.01 | GC ,MS:EI, Br+,KOH+ |
| 12 | 268 | *sec*-propyl tetradecenoate | 0.25 ± 0.15 | 0– 0.45 | 0.01 | GC ,MS:EI,CI, Br+,KOH+ |
| 13 | 266 | *sec*-propyl tetradecadienoate | 0.10 ± 0.06 | 0.05– 0.21 | 0–0.01 | GC ,MS:EI,CI, Br+,KOH+ |
| 14 | 282 | *sec*-butyl tetradecenoate | 0.40 ± 0.24 | 0.17– 0.71 | 0.01 | GC ,MS:EI,CI, Br+,KOH+ |
| 15 | 280 | *sec*-butyl tetradecadienoate | 0.43 ± 0.20 | 0.22– 0.72 | 0.01 | GC ,MS:EI,CI, Br+,KOH+ |
| 16 | 278 | *sec*-butyl tetradecatrienoate | 0.35 ± 0.18 | 0.17– 0.60 | 0.01 | GC ,MS:EI,CI, Br+,KOH+ |
| 17 | 124 | *p*-toluhydroquinone | 3.48 ± 4.88 | 0–14.09 | 0.20–0.45 | GC*,MS:EI,CI, Br–,KOH– |

[a]GC = separated by gas chromatography, *compound has similar retention time to the authentic compound; MS = mass spectrometry; EI = EI mass spectra (electron impact ionization) were recorded; CI = CI mass spectra were recorded (chemical ionization); HR = molecular formulas of M+ peaks and fragments have been determined by high-resolution measurements; Br– = component unaffected on treatment with bromine; Br+ = component removed from GC profile on treatment with bromine; KOH+ = component removed from GC profile on treatment with potassium hydroxide; KOH– = component unaffected on treatment with potassium hydroxide.

41, 42, and 59 amu, while there were losses of 55, 56, and 73 amu for compounds 2, 3, and 7. The fragments at M-41 and M-42 have been shown to result from the loss of a propyl, and those at M-55, M-56 from the loss of a butyl residue from an ester moiety, together with transfer of two hydrogen atoms to produce the carboxylic acid ion and the protonated carboxylic acid ion. Fragments at M-59 and M-73 constitute acylium ions. Compounds 2, 3, 4, and 7 were unaffected on treatment with bromine but were removed from the GC profile on treatment with aqueous potassium hydroxide (Table 1). EI mass spectral data and a comparison of retention times showed that compound 4 of the gland system 1 of *D. dichrous* is isopropyl dodecanoate (*sec*-propyl dodecanoate). Like authentic isopropyl dodecanoate compound 4 of *D. dichrous* is characterized by a weak fragment at *m/z* 61 and a distinct peak at *m/z* 200. On the other hand, authentic *n*-propyl dodecanoate reveals a base peak at *m/z* 61 and shows weak fragment at *m/z* 200 as seen in a detailed mass spectroscopic comparison of propyl esters (Dettner, 1984). Moreover, isopropyl dodecanoate (4) of *D. dichrous* showed distinctly shorter retention times on a 8-m CW 20 M capillary column as compared with authentic *n*-propyl dodecanoate (compound 4 from *D. dichrous*: 1100 sec; authentic isopropyl dodecanoate: 1104 sec; authentic *n*-propyl dodecanoate: 1234 sec).

EI mass spectra and retention values of constituents 2, 3, and 7 revealed that *D. dichrous* sequesters *sec*-butyl decanoate (2), *sec*-butyl undecanoate (3), and *sec*-butyl dodecanoate (7) in its red defensive glands. EI mass spectra of the three beetle constitutents show a base peak at *m/z* 57 which was found to be characteristic of authentic *sec*-butyl esters of $C_{10}$, $C_{11}$, and $C_{12}$ carboxylic acids, whereas a fragment at *m/z* 56 constitutes the base peak in the corresponding authentic isobutyl and *n*-butyl esters (Figure 8). Moreover authentic *sec*-butyl esters and compounds 2, 3, and 7 from *Deleaster* are characterized by distinct M-56 fragments as compared with the relatively intense M-55 fragment. Isobutyl and *n*-butyl esters of the above-mentioned acids showed only minute M-56 fragments when compared with the M-55 peaks (relative intensities of ester fragments M-56: M-55: *Deleaster* 2, 3, and 7: 0.67–0.79; authentic *sec*-butyl esters: 0.64–0.77; isobutyl esters: 0.36–0.44; *n*-butyl esters: 0.14–0.21). The *Deleaster* esters 2, 3, and 7, the three authentic *sec*-butyl esters, and *n*-butyl esters were finally characterized by a distinct M-29 fragment whereas isobutyl esters showed a fragment at M-30 (Figure 8). On a 8-m CW 20 M capillary column natural and authentic *sec*-butyl esters were found to possess the shortest retention times when compared with *n*-butyl and isobutyl esters ($R_t$ dodecanoates: *Deleaster* compound 7: 1219 sec; authentic *sec*-butyl ester: 1218 sec; isobutyl ester: 1271 sec; *n*-butyl ester: 1328 sec).

*Unsaturated Esters (5, 6, 8–16)*. EI mass spectra of compounds 5, 6, 8–16 (Figure 7) from gland system 1 of *D. dichrous* showed molecular masses of 240 (5, 6), 254 (8, 9), 252 (10), 268 (11, 12), 266 (13), 282 (14), 280 (15), and 278

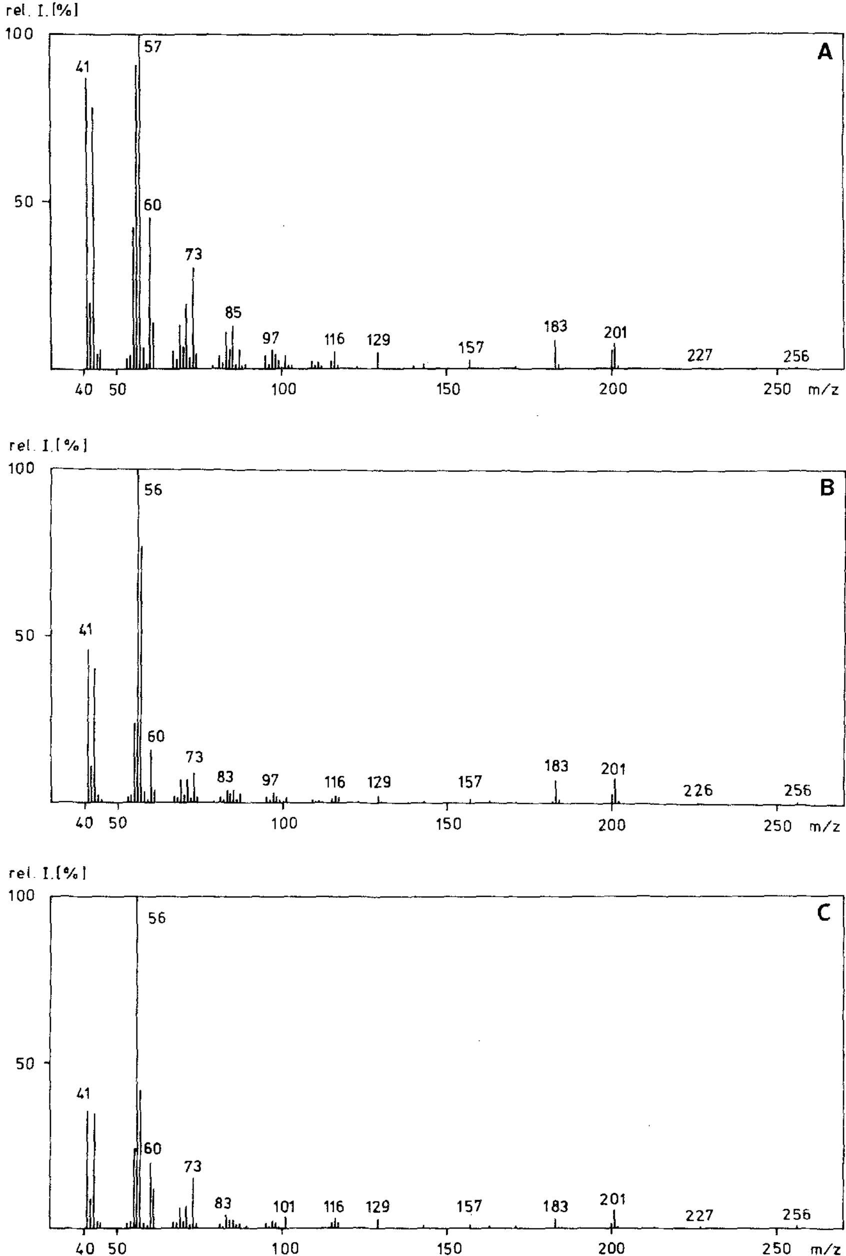


FIG. 8. EI mass spectrum of compound 7 (*sec*-butyl dodecanoate) from the red gland system of *Deleaster dichrous* (Grav.) (A) as compared with EI mass spectra of authentic isobutyl dodecanoate (B) and *n*-butyl dodecanoate (C).

(16), which were supported by M + 1 quasimolecular ions (CI-MS) for constituents 6, 8–10, and 13–16 (Table 1). The molecular ions of constituents 5, 6, 12, and 13 in each instance lose 42, 43, 59, and 60 amu, which has been shown to be characteristic for unsaturated isopropyl esters (Dettner, 1984). Compounds 8–11 and 14–16 were characterized by distinct fragments at M-56, M-55, M-73, and M-74 in the upper mass region of their EI mass spectra as was observed in authentic unsaturated *sec*-butyl esters. The presence of an ester function and unsaturation in the compounds mentioned previously (5, 6, 8–16) was supported when they were removed quantitatively from the GC profile on treatment with a solution of aqueous potassium hydroxide or bromine (Table 1). Components 10–16 therefore have been found to represent *sec*-butyl dodecadienoate (10), *sec*-butyl tridecenoate (11), *sec*-propyl tetradecenoate (12), *sec*-propyl tetradecadienoate (13), *sec*-butyl tetradecenoate (14), *sec*-butyl tetradecadienoate (15), and *sec*-butyl tetradecatrienoate (16). By carefully performing EI mass spectra, high-resolution measurements, epoxidations, and measurements of retention times, it was possible to determine position and configuration of the double bonds of the four major esters (Dettner and Schwinger, in preparation) biosynthesized by *Deleaster dichrous*. These compounds were identified by a combination of several microtechniques from the crude beetle secretion as: *sec*-butyl (Z)-3-dodecenoate (9), *sec*-propyl (Z)-3-dodecenoate (6), *sec*-butyl (Z)-5-dodecenoate (8), and *sec*-propyl (Z)-propyl (Z)-5-dodecenoate (5).

*Nonvolatile Defensive Secretion of Gland System 1*

When pure secretion free from tissue of the reddish gland system of *D. dichrous* was treated with a silylation (MSTFA) or a methylation ($BF_3$/MeOH) agent, there appeared several new peaks of the GC profile. To prevent transesterification of the naturally occurring isopropyl and *sec*-butyl esters, esterification was performed by using ethereal diazomethane solution. On the GC profile there appeared ten (Figure 9) additional new compounds (18–27) which were identified as methyl esters of carboxylic acids on grounds of their EI mass spectra. Both EI mass spectra and retention values of compounds 18 and 24 were identical with authentic methyl dodecanoate (18) and methyl hexadecanoate (24). Other methyl esters have been shown to be characterized by at least one double bond. Constituents 19 and 20 were identified as methyl dodecenoates ($M^+$: 212), 21 as methyl tetradecenoate ($M^+$: 240), 25 as methyl hexadecenoate ($M^+$: 268), 26 as methyl octadecenoate ($M^+$: 296), and 27 as methyl octadecadienoate ($M^+$: 294). Constituents 26 and 27 exhibited identical EI mass spectra when compared with authentic methyl esters of oleic acid (26) and linoleic acid (27). No molecular masses could be recorded for constituents 22 and 23. EI mass spectra and retention times are in favor of two methyl esters of tetradecenoid acid. Retention times and mass spectra of available straight-chain methyl dodecenoates were compared with $R_t$ values and MS data of constituents 19 and 20

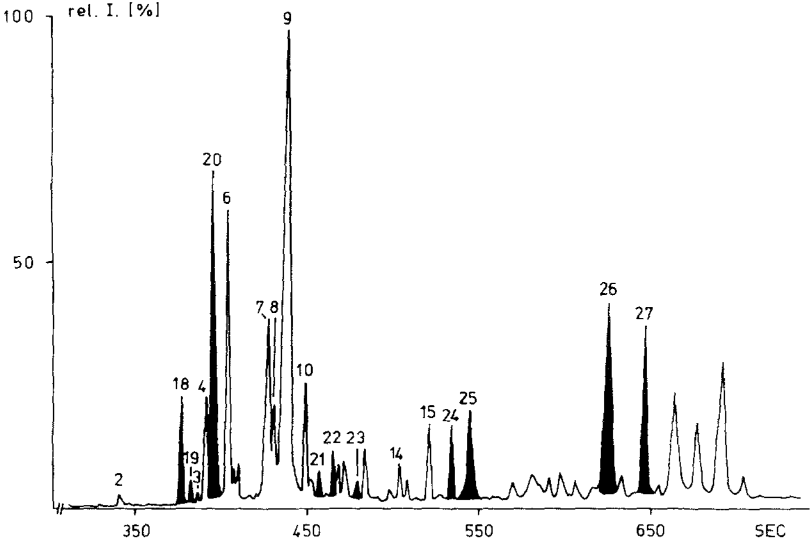


FIG. 9. Part of the total ion current chromatogram of the ethereal gland secretion of *D. dichrous* treated with diazomethane. Free fatty acids are characterized as methyl esters (black peaks 18–27; for further explanation see text).

(Dettner and Schwinger, in preparation), and it was finally suggested that the free dodecenoic acids show a *cis* configuration and have their double bonds located in a 3- (20) or a 5- (19) position. It seems highly probably that the free acids (19) and (20) biogenetically represent precursors for the main ester constituents *sec*-propyl (*Z*)-3-dodecenoate (6) and *sec*-butyl (*Z*)-3-dodecenoate (9).

### *Chemistry of Gland System 2 (White Gland)*

The white gland system 2 of *D. dichrous* is filled with a whitish liquid which hardens within some seconds on exposure to the air. There are great difficulties in isolating uninjured gland reservoirs 2 from the adhering gland reservoir 1. After dissection, the usually hardened secretion of gland system 2 did not react with 2,4-dinitrophenylhydrazine at room temperture but did so when heated in a closed glass capillary. At 200°C the secretion produced an orange coloration with 2,4-dinitrophenylhydrazine. When separated on a 8-m CW 20 M capillary column, gland 2 secretion was shown to consist of four main components 28–31 (Figure 10) which exhibited molecular masses at *m/z* 168. This was clearly confirmed by quasimolecular ions of 169 (M + 1; CI mass spectrometry). The four main components 28–31 represent between 11.3% and 81% of the total peak area found in the gas chromatographic analysis of the white gland secretion of the beetle (Table 2). Constituents 28–31 have been shown to be removed from the GC profile on treatment with a silylation (MSTFA)

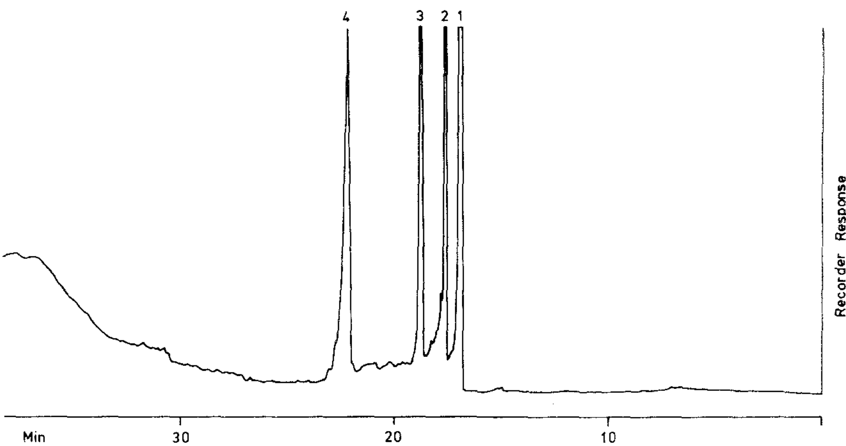


FIG. 10. Capillary gas chromatogram of two defensive gland reservoirs 2 (white glands) from *D. dichrous* by using a 8-m CW 20 M glass capillary. Constituents 28–31 represent four stereoisomeric iridodials (for further explanation see Table 2).

agent or with trifluoroacetic anhydride (TFAA). On the basis of this and the formation of a 2,4-dinitrophenylhydrazone, constituents 28–31 were tentatively described as bifunctional molecules with an aldehyde and alcohol function.

EI mass spectra of 28–31 were very similar and showed a typical fragment at *m/z* 81 which is common to all methylcyclopentane monoterpenoids (Regnier, 1972). Further important fragments are found at *m/z* 150 and 135. By high-resolution measurements of the filled gland reservoirs, and formula $C_{10}H_{16}O_2$ could be found (measured: 168.11613, calculated: 168.1150) for the components with molecular mass 168. The molecular ion loses $H_2O$ to produce fragments at *m/z* 150 (formula: $C_{10}H_{14}O_1$; measured: 150.1052, calculated: 150.1045). The fragment at *m/z* 135 was found to be characterized by the formula $C_9H_{11}O_1$ (measured: 135.0808, calculated: 135.0809). Constituents 28–31 were identified as stereoisomeric iridodials on the grounds of EI mass spectral data (Table 2) as compared with data from Bellas et al. (1974). Moore and Brown (1979), Smith et al. (1979), and Jefson et al. (1983). The replacement of the reported base peak at *m/z* 81 by a base peak at *m/z* 58 (see Table 2, 28–30) may be due to varying mass spectrometric conditions. EI mass spectral data of constituent 30 were in accordance with iridodial from *Ontholestes murinus* (L.) or *Philonthus carbonarius* (Gyll.) (Dettner, 1983).

It is well known that the pure iridodial readily polymerizes to paste, butter, or glasslike masses when the liquid is exposed to light or air (Cavill et al., 1956; Trave and Pavan, 1956; Pavan and Trave, 1958). The already described depolymerizing of iridodial was apparent when the hardened contents of the white gland were introduced into the hot injector of the capillary gas chromatograph

TABLE 2. COMPOSITION OF DEFENSIVE GLAND SYSTEM 2 (WHITE GLAND) OF *Deleaster dichrous* WITH FOUR STEREOISOMERIC IRIDODIALS AND ANALYTICAL EVIDENCE FOR ASSIGNMENTS AND EI MASS SPECTRAL DATA[a]

| Number | Molecular mass | Mean % peak area per beetle (± SD, $N = 5$) | Range of values found (% peak area, $N = 5$) | Evidence | MI-MS fragments (Relative abundance) |
|---|---|---|---|---|---|
| 28 | 168 | 27.5 ± 17.4 | 4.4–50.6 | GC, MS:EI, CI, MSTFA+, TFAA+ | 41(71), 43(45), 55(42), 58(100), 67(50), 71(38), 81(42), 97(11), 111(9), 122(5), 135(7.5), 150(4), 168(2.5) |
| 29 | 168 | 13.3 ± 8.0 | 3.1–22.4 | GC, MS:EI, CI, MSTFA+, TFAA+ | 41(95), 43(81), 55(64), 58(100), 67(56), 71(52), 81(72), 93(24), 111(11), 121(10), 135(16), 150(8), 168(4) |
| 30 | 168 | 10 ± 4.7 | 2.6–15.4 | GC, MS:EI, CI, MSTFA+, TFAA+ | 41(83), 43(63), 55(63), 58(100), 67(51), 81(74), 93(17), 109(30), 111(15), 135(14), 150(5), 168(3) |
| 31 | 168 | 9.3 ± 4.7 | 1.2–13.4 | GC, MS:EI, CI, MSTFA+, TFAA+ | 41(100), 43(90), 55(52), 58(59), 67(42), 71(55), 81(46), 97(34), 108(22), 11(21), 135(25.5), 150(6), 168(18). |

[a]GC = separated by gas chromatography; MS = mass spectrometry; EI = EI mass spectra were recorded; CI = CI mass spectra were recorded; MSTFA+, TFAA+ = component removed from the GC profile on treatment with MSTFA or TFAA (see text).

and when the secretion produced a 2,4-dinitrophenylhydrazone after, but not before, heating.

### *Gland Discharge and Biological Observations*

When caged with conspecifics, *Deleaster dichrous* responds only at distances less than 2–3 cm by bending its abdominal tip dorsally. Discharge of secretion directed against conspecifics was never observed. On contact with *Drosophila melanogaster*, the rove beetle *Deleaster dichrous* immediately bends its abdomen dorsally to touch the fly for a moment. Afterwards, the flies show an intensive cleaning behavior and die 1–2 hr later. Against ants of the genus *Myrmica, D. dichrous* only responded when its legs or antennae had been seized. Several ants characteristically showed an immovable, somewhat solidified antenna when this body organ had contacted the beetle's abdominal tip. On the basis of the these observations, it seems obvious that the secretion of gland 2 functions as an adhesive as it polymerizes on the body of small attacking arthropods. Simultaneously, secretion of gland system 1 must be mixed with the whitish gland contents of the gland system 2 after molestation and discharge of the glands. This could be clearly demonstrated when a coverglass fragment was applied to an abdominal tip of a *D. dichrous* specimen. Under the microscope, a small portion of hindgut material was found to be surrounded by a reddish fluid that polymerized to a butter-like material 30 sec afterwards. Moreover, single beetle specimens were molested once per hour (1–3 molestations per specimen), and the small microdroplets of defensive secretion were collected on a tiny triangle of filter paper which was directly injected into the gas chromatograph. Three successive molestations of the same specimen of *D. dichrous* clearly showed that the whole defensive exudate represents a mixture from both gland systems 1 and 2 (Figure 11).

Molestation experiments using six specimens of *D. dichrous* revealed extremely different percentage rates of gland depletion. A specimen of *D. dichrous* with completely filled gland reservoirs discharged 2.5% of its gland reservoir 1 and simultaneously 5% of its gland reservoir 2 when it was gently touched on its thorax with a forceps. Another beetle with partly emptied gland reservoirs exuded 44.6% (gland reservoir 1) and 74.6% (gland reservoir 2) of its sequestered secretion following touching with a forceps. Probably the amount of gland depletion depends to some extent on the intensity of the irritation; on the other hand, the defensive potential emitted during single and successive secretory acts depends mainly on the available glandular products remaining. Usually the ratio of depleted secretion 1 (gland 1) to secretion 2 (gland 2) was comparable with the ratio of sequestered secretion 1 to secretion 2. As shown in Figure 11, the proportion of defensive secretion of gland system 2, as compared with that of gland 1, decreased from molestation to molestation. This is probably due to the

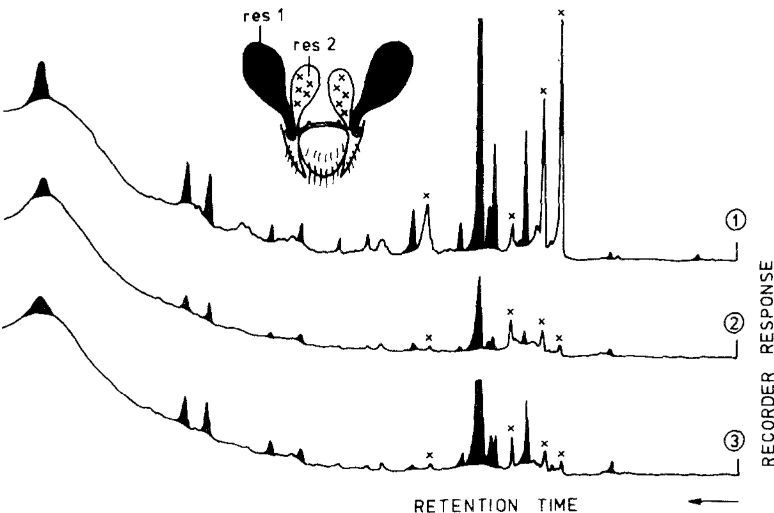


FIG. 11. Capillary gas chromatogram of three successive gland depletions of a thrice irritated specimen of *D. dichrous*. After each molestation, exuded defensive secretion was collected from the abdominal tip by using a tiny filter paper which was directly injected into the GC. Components from gland system 1 are characterized by black colors (black gland reservoir and peaks); simultaneously depleted iridodials from gland system 2 are indicated by crosses (gland reservoir, peaks).

low viscosity of iridodial (gl 2) as compared with the highly viscous secretion of gland 1. In every specimen investigated, the secretion of the red gland system was found to be saturated with *p*-toluquinone (approximately 3$\mu$g TQ per beetle).

To test the capability of some beetle solvents in ensuring the penetration of considerable amounts of the toxic *p*-toluquinone through chitin surfaces, *Lucilia* larvae were topically treated with droplets (1 $\mu$l) of different Oxytelinae-derived solvents saturated with toluquinone (Figure 12). Both main constituents of the *Deleaster* gland system 1 were almost ineffective. Larval mortality from a saturated solution of *p*-toluquinone in *sec*-butyl (*Z*)-3-dodecenoate became obvious only one week later. If isopropyl (*Z*)-3-dodecenoate was used as solvent, only 2 larvae of *Lucilia* died within 2 hr after the experiment. The efficiency of isopropyl (*Z*)-3-dodecenoate was therefore comparable to isopropyl dodecanoate, which is present as the major component in the defensive gland of *Coprophilus striatulus*, another primitive species of the Oxytelinae. Figure 12 clearly shows that the 1-alkenes of the taxonomically derived Oxytelinae species (Dettner and Schwinger, 1982) are highly effective as compared with other solvents of the primitive genera *Coprophilus* (isopropyl dodecanoate) or *Deleaster* [isopropyl (*Z*)-3- and *sec*-butyl (*Z*)-3-dodecenoate].

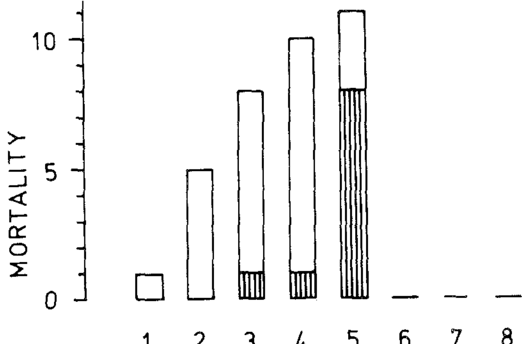


FIG. 12. Single determinations of mortalities of *Lucilia* larvae 2 hr (hatched area of columns) and four days (columns) after treating topically with 1 $\mu$l solution ($N$ = 20 larvae tested per solution). 1: untreated control; 2–6 with *p*-toluquinone saturated solutions; solvents: 2, *sec*-butyl (*E*, *Z*)-3-dodecenoate; 3, isopropyl (*E*, *Z*)-3-dodecenoate; 4, isopropyl dodecanoate, 5, 1-undecene; 6, $\gamma$-dodecalactone; 7 and 8: solvents without quinone: 7, 1-undecene; 8, $\gamma$-dodecalactone.

## *Chemotaxonomy*

Within the 1700 species of the worldwide subfamily Oxytelinae, the genus *Deleaster* shows many phylogenetically primitive characters. According to various investigators (e.g., Herman, 1970; Newton, 1982), the seven species of the genus *Deleaster* represent the most primitive Oxytelinae species currently known. *Deleaster* species also show some characteristics of the subfamily Omaliinae such as only one abdominal laterosternite and a poorly scelerotized second abdominal sternite. On the other hand, the presence of defensive glands whose external openings are confined to the ninth tergite would place the genus *Deleaster* in the Oxytelinae. The assumed phylogenetic antiquity of *Deleaster* is supported by the relict distribution of this genus. The chemical inventory of gland system 1 of *D. dichrous* differs completely from the defensive constituents of the homologous abdominal defensive gland of phylogenetically derived oxyteline species.

Derived and primitive Oxytelinae species share only the common presence of *p*-toluquinone, the toxic principle of the typically oxyteline abdominal gland. Phylogenetically derived species are characterized by the presence of long-chain $\gamma$-lactones and corresponding 1-alkenes. It has been suggested that both constituents, the 1-alkene and the lactone of the derived Oxytelinae, could be formed from a $\beta$, $\gamma$-unsaturated acid either by decarboxylation or by addition across the double bond (Wheeler et al., 1972; Figure 13). This biogenetic suggestion is supported by the quantitative ratio of several 1-alkenes to $\gamma$-lactones within all derived species investigated (Dettner and Schwinger, 1982). It is fascinating that the supposed precursor (*Z*)-3-dodecenoic acid, for the defensive solvents $\gamma$-do-

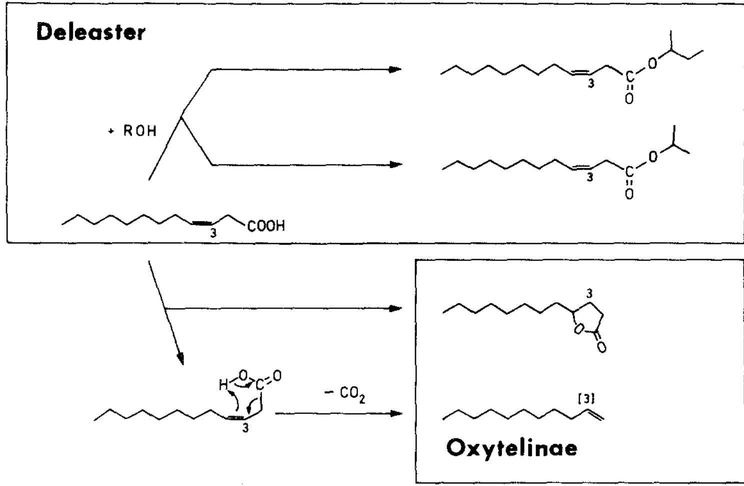


FIG. 13. Evolutionary trends with respect to the evolution of the solvents of the quinoid oxyteline defensive gland. γ-Lactones and corresponding 1-alkenes from the phylogenetically derived Oxytelinae must originate from a 3,4-unsaturated acid which is exclusively present within the glands of the primitive species *Deleaster dichrous* both as free acid and as ester.

decalactone and 1-undecene (Figure 13) of the derived species is probably present as main constituent (20, Figure 9) in defensive gland 1 of the primitive genus *Deleaster*. Probably because of the high melting point of this unsaturated acid, *Deleaster dichrous* synthesizes the appropriate isopropyl and *sec*-butyl esters as main components (6, 10) in its defensive gland system 1 (Figures 7 and 9).

## DISCUSSION

The rove beetle *Deleaster dichrous* probably represents the first beetle species characterized by a complex gland with a sticky secretion. Exudates from arthropod exocrine glands which become very viscous after being discharged are mainly found within termites, aphids, and ants (see Blum, 1981; Eisner, 1970). Sticky secretions of termites are sometimes augmented by cooccurring benzoquinones (Blum, 1981). Iridodial-containing anal glands of the dolichoderine ant *Tapinoma nigerrimum* also contain carbonyl compounds (Trave and Pavan, 1956). *Deleaster dichrous* therefore is unique in its morphological separation of two gland reservoirs containing pure monomeric adhesive (glands 2) on the one hand and two further reservoirs on the other hand where a deterrent quinoid secretion is stored (glands 1).

The joint utilization of these nonhomologous glands 1 and 2 after a molestation is fascinating. With respect to the ant *Tapinoma nigerrimum*, Pavan and

Trave (1958) suggested that the rapidly polymerizing iridodial might function as a fixative for the more volatile carbonyl compounds stored in the same gland system. This seems improbable in the case of *D. dichrous* where the ester solvents of the gland system 1 are nonvolatile or only slightly volatile because of the length of their carbon chain. It seems probable that iridodial of gland system 2 functions exclusively as an entangling agent against small predatory arthropods since this dialdehyde exhibits no insecticidal properties (Blum and Hermann, 1978). Additional functions of the four gland system of *Deleaster* comparable with those found in pygidial gland secretions of water beetles (Dettner, 1985) are possible. However, it seems highly improbable that a beetle would simultaneously exude a complex mixture of defensive compounds from four reservoirs, after a molestation for any other purpose than chemical defense.

Nevertheless, the precise predatory target organisms of the four-gland chemical defensive system of *D. dichrous* are not known. *D. dichrous* is a rare species, usually found under rocks along cold unpolluted streams. The species flies readily and may easily be caught at light traps during the night. In the Eifel region, the species was found in wet gravel (only one locality) near the river edge. Laboratory caged specimens have been found to construct tunnels within the wet fine granular gravel. Caged beetles fed on freshly killed *Calliphora* flies, which were often transported into the tunnels. *D. dichrous* also fed on parts of *Bellis*, *Taraxacum*, and *Ranunculus* blossoms. The habitat of *D. dichrous* somewhat resembles the biotope of nonhalophile *Bledius* species of the same subfamily. These *Bledius* species, although also capable of constructing subterran burrows, prefer sandy substrates and feed on algae. A subsocial life history as known in *Bledius* or *Platystethus* species (Hinton, 1944) may also occur with *Deleaster* since several specimens of the latter are usually found together, under rocks or within their subterranean burrows. Several species of hydrophilic carbids were the only arthopods to be found in the same wet habitat together with *D. dichrous*.

The red glands of *Deleaster dichrous* so far represent the first defensive glandular source in insects for *sec*-butyl esters. Only a few *n*-butyl esters and one isobutyl ester are known from metathoracic scent glands of Heteroptera (Aldrich and Yonke, 1975; Baker et al., 1972). The defensive glands of the rove beetle *Coprophilus striatulus* are rich sources for isopropyl esters (Dettner, 1984). Other insect species producing isopropyl esters are the Argentine ant *Iridomyrmex humilis* (Cavill and Houghton, 1974) and males of *Dermestes maculatus* whose esters act as pheromones (Francke et al., 1979). Finally *sec*-butyl decanoate, *sec*-butyl dodecanoate, *sec*-butyl (Z)-7-tetradecenoate, and isopropyl (Z)-7-tetradecenoate have been found as sex pheromone components of the western grape leaf skeletonizer *Harrisiana brillians* (Myerson et al., 1982). Isopropyl and *sec*-butyl esters are recognized as excellent wetting agents and are used in cosmetic and topical medicinal preparations where good absorption

through the skin is desired. Immersion experiments by using *Lucilia* (Figure 14) or *Calliphora* (Dettner, 1984) larvae, however, demonstrated that the efficiency of the quinoid solutions depends on good wettability of the defense solution on arthropod cuticle.

The chemical complexity of its defensive secretions seems inconsistent with the suggested primitive status of *Deleaster.* This genus is unique in possessing the sticky defensive system 2, which is absent from other members of the Oxytelinae and rove beetle species from other subfamilies. On the other hand, a primitive position for *Deleaster* within Oxytelinae is supported if its quinoid gland system 1 is compared with the homologous defensive gland of other members of the subfamily.

Morphologically, gland reservoir 1 of *Deleaster* is small compared with the body volume. In highly evolved oxyteline species, there is a tendency to develop larger glandular reservoirs, probably because this ensures adequate defense against repeated molestations. Further primitive states of gland system 1 of *Deleaster* comprise the short efferent duct (which is lengthened in highly evolved species) and the openings of gland reservoirs 1 situated near the anterior border of the ninth tergite. Openings of the defense glands of derived species are usually situated centrally on the ninth tergites. Finally, cuticular ductules of the gland cells of *Deleaster* are broadened and shortened, in contrast with the narrow and lengthened ductules of more evolved species such as *Bledius.*

The defense chemistry of gland system 1 of *Deleaster dichrous* in comparison with that of other Oxytelinae represents the first case within insects where a clear molecular evolutionary trend can be discerned from primitive towards highly evolved species parallel to that apparent from morphological studies (Hermann, 1970; Newton, 1982). Although plausible phylogenetic phenoclines of ant alkaloids have been reported (Blum, 1981), the dendrogram of the hymenopteran genus *Solenopsis* investigated remains unknown. Other distinct micromolecular trends in mandibular gland secretions of the ant tribe Attini (Blum, 1981) and in abdominal defensive gland constituents of Tenebrionidae (Tschinkel, 1975) also illustrate the trends in evolution of new glandular constituents from primitive towards derived species.

If one assumes that the efficiency of a defensive secretion will be improved from phylogenetically primitive to derived species, gland system 1 of *D. dichrous* represents a good starting point. Although this reddish quinoid ester secretion exhibits good wettability characteristics on arthropod cuticles (as in *Coprophilus*; Dettner, 1984) and excellent solvency for the toxic *p*-toluquinone, the larval mortalities of *Lucilia* larvae were found to be extremely low in comparison with other solvents (e.g., 1-undecene) of highly evolved species. If it can be assumed that the results from the larval immersion experiments would be repeatable with other test organisms, the reason for *Deleaster dichrous* to evolve an effective sticky gland system 2 as a backup to its rather inefficient

gland system 1 immediately becomes clear. A plausible evolutionary trend with respect to the solvents of the defensive glands of all Oxytelinae is evident, if other species of this subfamily are compared (Dettner, Wunderle, and Schwinger, in preparation: It is presumed that free $\beta$, $\gamma$-unsaturated fatty acids within the range of $C_{10}$ to $C_{16}$ represented preadaptations for dissolving the toxic *p*-toluquinone (Figure 13). To prevent solidification of the gland contents from free $\beta$, $\gamma$-unsaturated acids, there were probably synthesized either isopropyl esters and *sec*-butyl esters or 1-alkenes via decarboxylation (Figure 13). In the derived species characterized by alkenes from the same unsaturated acid precursor, $\gamma$-lactones may have originated, which augment the repellency of the quinoid defensive secretions, but only if they occur in admixture with 1-alkenes (Dettner, in preparation).

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# LACK OF EFFECT OF (−)-DISPARLURE ON ORIENTATION TOWARDS (+)-DISPARLURE SOURCE IN WALKING AND FLYING GYPSY MOTH MALES

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**Abstract**—In order to reveal the mechanism by which an inhibitory substance acts, orientation towards an attractant and attractant–inhibitor mixed source was studied in male gypsy moths. The inhibitory (−)-disparlure had no effect on the moth's ability to locate the attractant (+)-disparlure source while walking or flying in a laminar airstream. This absence of any inhibitory effect indicates that only very specific components of the moth's orientation behavior, as discussed, are distributed by the (−)-disparlure.

**Key Words**—Gypsy moth, *Lymantria dispar,* Lepidoptera, Lymantriidae, sex pheromone, disparlure, enantiomers, anemotaxis, chemotaxis, inhibitor, flight orientation.

## INTRODUCTION

The attractant pheromone of the gypsy moth, *Lymantria dispar* L., has been described as *cis*-7,8-epoxy-2-methyloctadecane = "disparlure" (Bierl et al., 1970). Since this molecule is chiral, it forms *R,S* and *S,R* enantiomers, hereafter called (+)- and (−)-disparlure, respectively.

Field trapping experiments (Vité et al., 1976; Cardé et al., 1977) proved that (+)-disparlure is highly attractive to the moths, whereas an addition of (−)-disparlure lowers this attractivity. With racemic (±)-disparlure, trap catches were reduced to a tenth (Vité et al., 1976) due both to fewer moths starting to fly upwind and to fewer landing on the trap (Cardé et al., 1977). Its effect on landing was attributed to the reduced persistence of anemotactic flight (Miller and Roelofs, 1978; Cardé and Hagaman, 1979). This implies that the inhibitory (−)-effect is much greater on in-flight than on preflight behavior. Such an effect



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is also apparent in the blocking by (−)-disparlure of the reactions responsible for the stabilization of flight altitude and speed (Preiss and Kramer, 1983). In order to reveal further components of the moths' orientation behavior that may be influenced by (−)-disparlure, walking as well as flying approaches to disparlure sources were studied in choice situations.

## METHODS AND MATERIALS

Male *L. dispar* were reared in the laboratory from field-collected eggs. The larvae were fed acorns until the third instar, when they were fed spruce needles (*Picea abies*). Male pupae and eclosed male moths were kept in a 16-hr–8-hr light–dark cycle at room temperature (about 22°C) in cloth netting cages over water. Experiments were carried out with 24- to 36-hr-old males during the second half of the photophase when, under natural conditions, the moths are spontaneously active (Schröter, 1976). In experiments with walking moths, their wings were clipped to about 5 mm long at least 1 hr before the experiment started.

*Locomotion Compensator.* The moth walks on the "north pole" of a black-painted sphere that is rotated by motors in such a way as to compensate for the moth's translocation. The moth is thereby kept in place without restraining its locomotory activity (for technical details see Kramer, 1975, 1976). The sphere's movement, which represents the effective locomotion of the moth, is sampled every second and stored as $x$–$y$ coordinates on paper tape for further processing by computer. In addition the tracks were recorded directly on an $x$–$y$ plotter. Care was taken to ensure a homogeneous optical environment.

*Stimulus Condition.* An almost laminar airstream was blown from a constant direction (y direction) over the top of the sphere by a delivery system previously described by Kramer (1975) (outlet of the air 10 × 2 cm). The delivery system was halved into parallel channels separately supplied with air (wind speed in both channels 0.4 m/sec). The synthetic pheromone component, either (+)- or (−)-disparlure, or the 50:50 racemate, was applied on filter paper (1 × 3.5 cm) and positioned within one half of the divided air stream. In further experiments, differently loaded channels were used to test the attractiveness of the odors simultaneously. (The enantiomers were kindly supplied by Dr. K. Mori and their optical purity was over 98%; Mori et al., 1976).

Under these experimental conditions, a moth walking exactly upwind had its left and right antennae in the corresponding halves of the air stream. Only when deviating from this direction could it receive the same odor signal with both antennae. In order to eliminate any effects of unnoticed asymmetries in the stimulus situation, the filter papers were slipped every 50 sec from one channel to the other by means of a motor. Thus, any preference for one channel (compound) was expected to result in a zig-zag track in synchrony with these switchings. One experiment lasted 1000 sec (10 periods) without break.

*Flight Tunnel.* Approach flights towards one or two neighboring pheromone sources in a flight tunnel were video recorded from above and then analyzed by single-frame analyses.

Within the flight tunnel (115 × 50 × 50 cm), a flight chamber (80 cm length) was separated off; its side walls as well as the upwind and downwind front sides were covered with black porous paperfelt, the floor having a checkered pattern (size of each bright or dark square: 2 $cm^2$). Air was sucked through by means of a fan (air speed 0.3 m/sec measured in the center of the chamber), its outlet leading outside the building. Due to the small size of the pores of the paper felt, air which did pass through was practically laminar within the chamber, as indicated by substituting the pheromone source by a smoke source. The flight chamber was diffusely illuminated from above via an inclined semilucent mirror, which provided a top view of the flights from the side.

The attractive (+)-disparlure was applied on a filter paper (3.5 × 1 cm) and positioned within a glass tube, its outlet (0.5 cm diameter) touching the upwind screen of the flight chamber from outside. In choice experiments, a second glass tube with a filter paper loaded with (−)-disparlure was positioned at a distance of 6 cm from and alongside the first. In one experimental series, (−)-disparlure was absorbed on a cotton thread which was stretched vertically across the tunnel, outside the flight chamber, at a distance of 5 cm from and alongside the (+)-disparlure source (~1.5 μg (−)-disparlure/cm of thread). In this case an odor "pencil" of (+)-disparlure was paralleled by a "curtain" of (−)-disparlure within the tunnel, whereas with two filter paper sources two discrete odor pencils were formed.

Up to 10 males were placed within a cotton screen cage in front of the downwind screen of the flight chamber within the range covered by both pheromone sources. The cage was opened about 30 sec after bringing the pheromone sources into place, to make sure that the moths had already perceived pheromone before starting.

The approach flights were redrawn from the video screen on plastic sheets by marking the position of the moths after each 0.04-sec interval (two frames of the video tape). The number of markings within 2.5-cm-wide vertical slices parallel to the long axis of the odor plume was plotted over the distance from the axis. This histogram represents the probability of the moths being within a given distance of the central axis of the odor plume and thus the accuracy of their following of the plume.

## RESULTS

### *Walking Moths*

*Choice Between Odorous and Pure Air Channel.* In a first series of experiments, one of the channels was loaded with (+)-disparlure; and in a second series, with the racemic (±) mixture. In both cases the moths oriented upwind

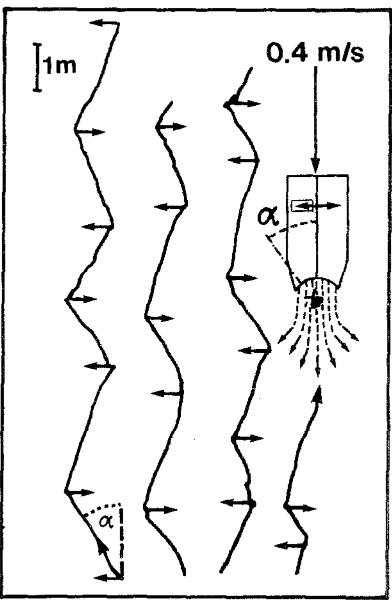


FIG. 1. Track of a moth walking upwind on the locomotion compensator against a double-channel airstream. Only one channel was loaded with 0.5 μ (+)-disparlure (inset). The odor-bearing channel was periodically changed (arrows).

with an angular deviation towards the odor-bearing channel (Figure 1). When the odor was slipped into the other channel, the moths immediately answered by reselection of the now odorous channel. The moths were thus zig-zagging in synchrony with these changes. Quantitative analysis of the tracks showed that it made no significant difference whether the "inhibitory" (−)-disparlure was admixed or not (Figure 2). In both cases walking speed (Figure 2A) and straightness of the tracks (Figure 2B) were the same and independent of the odor concentration (straightness = ratio of vector length from starting point to end point of a walk, over the course length; vector length was calculated from the data after taking the periodicity changed odor position into account). The moths' preference for the odorous channel (quantified by the number of 1-sec track vectors deviating towards the odorous channel) (Figure 2C) and their angular deviation from the upwind direction (Figure 2D) both increased with increasing concentration, and in the same way.

*Choice between Two Odorous Channels.* The moths were submitted to a sequence of choice situations (up to three repetitions with each of 15 moths). The test situations and the moths' preferences are given in Table 1; the source loading in all cases was 0.5 μg of the respective enantiomer. (1) The channel loaded with (+)-dispalure was always preferred, whether it was competing with a pure air or with a (−)-disparlure channel. (2) The channel loaded with (−)-disparlure was neither preferred nor avoided. (3) The channel loaded with

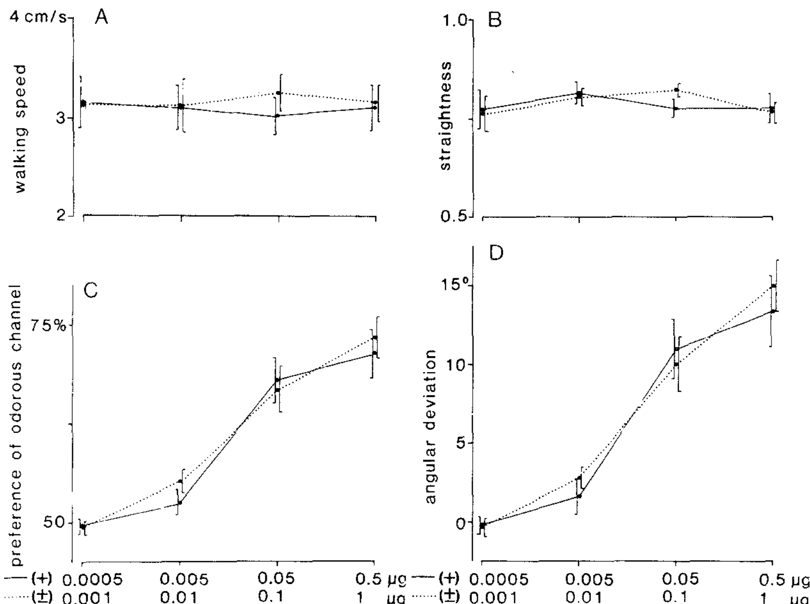


FIG. 2. Test situation as in Figure 1. One channel was loaded with either (+)-disparlure (solid line) or (±)-disparlure (dotted line). (A) Walking speed, (B) straightness of the tracks, (C) preference for the odorous channel, and (D) angular deviation from the upwind direction towards the odorous channel are not significantly altered in the presence of (−)-disparlure.

the racemic mixture was as attractive as the channel loaded with (+)-disparlure alone (no preference).

## *Flying Moths*

With a source of 0.5 $\mu$g (+)-disparlure on a filter paper, males flew upwind along the pheromone "pencil" on a convoluted track with deviations from the

TABLE 1. PREFERENCES FOR DISPARLURE ENANTIOMERS AND THEIR RACEMIC MIXTURE (0.5 $\mu$g OF EACH ENANTIOMER) UNDER CHOICE SITUATIONS IN WALKING MOTHS

| Left channel | Preferred channel | Right channel |
|---|---|---|
| (+) | ← | pure air |
| (+) | ← | (−) |
| (−) | ↑ | pure air |
| (±) | ↑ | (+) |

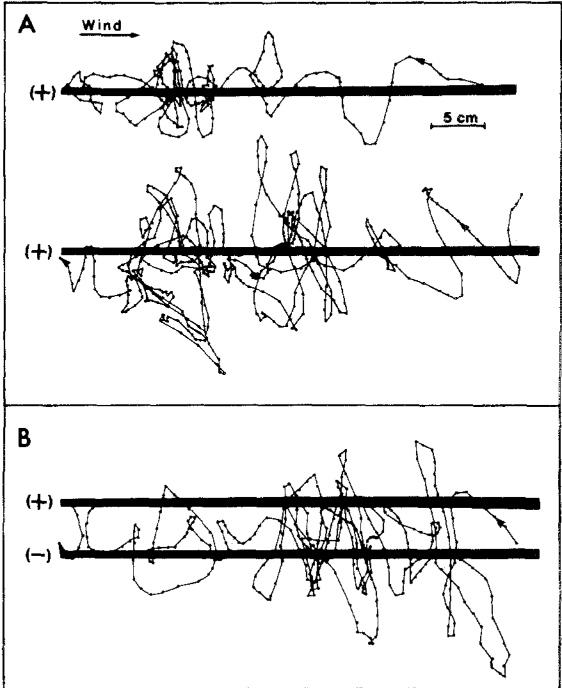


FIG. 3 Flight tracks of three moths orienting along (A) (+)-disparlure filament or (B) (+)-disparlure filament paralleled by a (−)-disparlure curtain. The moths' positions are marked every 0.04 sec (dots). The horizontal bar represents the odor.

central axis of the plume in both the horizontal and the vertical planes. Although for technical reasons only the deviations in the horizontal plane were recorded and evaluated (Figure 3A), it was obvious by observing the approach flights that the deviations in the vertical plane were similar. The histogram of the moths' whereabouts in the horizontal plane gave a Gaussian density distribution, symmetrical with respect to the pheromone line (Figure 4). When this (+)-disparlure pencil was paralleled by a curtain of (−)-disparlure 5 cm away within the tunnel, the approach flights were shifted away from the (+)-disparlure pencil (Figure 3B). The distribution of the corresponding histogram was widened, its peak now coinciding with the position of the (−)-disparlure curtain (Figure 4).

Since this surprising "attractivity" of the (−)-disparlure was suspected to be in fact its 2% (+)-disparlure content [amounting to 0.03 $\mu$g (+) per cm of thread], a 0.05-$\mu$g (+)-disparlure filter paper source was tested as a control (the evaporation rate from cotton is higher than that from filter paper; Kanaujia, in preparation). This source was also strongly attractive to the males (Figure 4). Since the probability of a moth losing contact with the pheromone by flight maneuvers in the vertical plane is reduced in the case of a curtain, both enan-

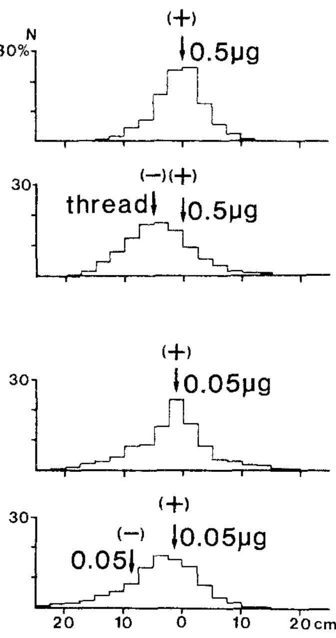


FIG. 4. Histograms of the moths' whereabouts with respect to the longitudinal axis of the odor plumes (arrows).

tiomers (0.5 μg each) were tested on filter paper. The corresponding histogram was single peaked; its peak now coincided with the position of the (+)-disparlure plume, although slightly shifted towards the (−)-disparlure plume. This, again, might have been caused by the attractivity of the 2% (+) content of the (−)-disparlure.

## DISCUSSION

While the mechanism of flight orientation towards an attractant odor source is basically understood, the mechanism by which an inhibitory substance acts on the orientation process is still a mystery. During the last 10 years, most work dealing with inhibitors has been focused on the reduced efficacy of traps in the field, if the inhibitor was emitted together with the attractant. This work, however, hardly touched the underlying mechanism.

According to Roelofs' (1978) threshold hypothesis of pheromone perception, the efficacy of an attractant can be reduced by an inhibitory compound both by raising the moth's activation threshold and by lowering the upper threshold where flight is altered due to an overdose of attractant stimulus (disorientation). It appears that the (−)-disparlure acts on both thresholds. A rise of the

activation threshold was found in field experiments (Cardé et al., 1977) and flight tunnel tests (Miller and Roelofs, 1978) where the percentage of moths initiating anemotactic flight was reduced. An interference with the orientation process was found in tethered flight experiments (Preiss and Kramer, 1983). Reactions which in free flight are responsible for the stabilization of flight altitude and speed were blocked.

In the experiments reported here, the expected inhibitory influence of (−)-disparlure was found neither with walking nor with flying moths. Its influence on the moths' ability to locate an odor source by anemotaxis, modulated by chemotaxis, was tested with walking moths. The moths, however, were able to orient towards a (+)/(−)-disparlure mixed source; in choice experiments the (+)-disparlure and the (±)-disparlure sources were equally attractive.

Due to the structure of an odor plume (Murlis and Jones, 1981), however, orientation of a flying moth locating an attractant source in the field cannot depend on this mechanism, but rather depends on a "pheromone-modulated optomotor anemotaxis" (Kennedy and Marsh, 1974; Marsh et al., 1978). The possible effect of (−)-disparlure on this optomotor mechanism was tested in flight tunnel experiments. The (−)-disparlure, however, again was totally ineffective as an inhibitor but rather seemed to be attractive by itself. This attractivity, however, could be explained by its pollution with the antipode. Although the pollution was less than 2%, the (+)-disparlure's attractivity was not blocked by the (−)-disparlure. All results, therefore, indicate that under these experimental conditions (−)-disparlure was totally ineffective just as if it were not present.

Since there is an inevitable mutual pollution of the disparlure enantiomers with their antipode due to synthesis, this prevents us from measuring the moth's orientation behavior induced by (+)-disparlure alone. Only the influence of an increased amount of (−)-disparlure can be studied. Since the inhibitory effect of admixed (−)-disparlure was found in field experiments (Vité et al., 1976) and tethered flight experiments (Preiss and Kramer, 1983), contamination of the test apparatus cannot explain the lack of inhibitory effect of (−)-disparlure in these new experiments, although care was taken to prevent this. The effect induced by contamination would be much smaller than the effect induced by the ever-present pollution. The results rather favor the idea that the observed inhibitory power of (−)-disparlure in the field experiments is attributed to its ability to block only very specific components of the moth's orientation behavior, but not the whole orientation process as implied by Roelofs' (1978) threshold hypothesis.

It follows that these specific components are expected not to be essential for the moths when orienting towards an odor source while walking as well as flying within a rather laminar airstream, as in this flight tunnel. Under field conditions, however, the moths, in addition, have to stabilize their flight against

wind turbulences, which are always present. As found in tethered flight experiments, the reactions responsible for the stabilization of flight altitude and speed against wind disturbances are blocked by the (−)-disparlure (Preiss and Kramer, (1983). Although this effect of (−)-disparlure seems to be rather subordinate, under field conditions it would play an important role and might explain the reduced trap catches (Vité et al., 1976). The reduced duration of anemotactic flight as found in a sustained-flight tunnel (Miller and Roelofs, 1978) might possibly be explained by this effect too. Although the air flow was smoothed, wind disturbances might be unintentionally simulated by countermoving the floor pattern with inadequate dynamics when keeping the flying moths in place. The data presented here, however, do not exclude that this reduced flight persistence is due to an additional effect of (−)-disparlure in lowering the moth's motivation to fly towards the source in the presence of (−)-disparlure.

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# VOLATILES FROM MANDIBULAR GLANDS OF MALE BEEWOLVES (HYMENOPTERA: SPHECIDAE, *Philanthus*) AND THEIR POSSIBLE ROLES

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**Abstract**—Observations of wasp behavior indicate that male beewolves, *Philanthus basilaris* and *P. bicinctus* apply a "marking pheromone" to the leaves and stems of plants within their territories. We now provide direct evidence for the presence of volatile chemicals in the paired mandibular glands of the males, provide preliminary identification of these volatiles, and show that all of the volatile chemicals in the mandibular glands are present on freshly marked plant surfaces but are absent from unmarked plants. Pyrazines, which have been reported in other species of aculeate wasps including the European *Philanthus triangulum*, were not found in *P. basilaris* or *P. bicinctus*.

**Key Words**—*Philantus*, beewolf, Sphecidae, Hymenoptera, pheromones, scentmarking, tridecanone, pentadecanone, heptadecanone, hexadecanoic acid, ethyl hexadecanoate, octadecanoic acid, ethyl octadecanoate, mandibular glands, wasps.

## INTRODUCTION

Beewolves are digger wasps in the genus *philanthus* (Sphecidae). Males within this genus display a behavior referred to as "abdomen-dragging"; they alight



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on plants within their territories and walk with their mandibular area and abdominal venter pressed against a stem or leaf (e.g., Alcock, 1975; Borg-Karlson and Tengö, 1980; Evans, 1983; Gwynne, 1978; O'Neill, 1979, 1981, 1984). Morphological evidence suggests a scent-marking function for this behavior. Males have hair brushes on the clypeus (at the base of the mandibles) and on the venter of the abdomen (Gwynne, 1980; O'Neill, 1979). They also have large mandibular glands which have been postulated to provide the source of exocrine secretions thought to be deposited via the mandibular hair brushes (Gwynne, 1978; O'Neill, 1979). Females do not have large mandibular glands in any of the species that have been examined, nor are they large in the males of *P. albopilosus,* a species that does not exhibit abdomen-dragging (Gwynne, 1978; O'Neill, unpublished). A similar correspondence between behavior and gland size has been reported in bees of the genus *Centris* (Raw, 1975).

Despite the strong circumstantial evidence outlined above suggesting that some *Philanthus* males are depositing a pheromone on the plant while abdomen-dragging, no direct evidence for this phenomenon has been reported. We now report preliminary identification of volatiles from *Philanthus basilaris* and *P. bicinctus,* demonstrate that these volatiles originate in the paired mandibular glands of the males, and that all of the volatiles contained in the glands are deposited onto the plant surface by the males during the behavior known as abdomen-dragging. A detailed chemical structural analysis of all of these chemicals will be presented in a subsequent paper (McDaniel et al., in preparation) with a brief description of the chemical method presented here.

## METHODS AND MATERIALS

*Biological. Philanthus basilaris* Cresson and *P. bicinctus* (Mickel) were collected and studied at the Great Sand Dunes National Monument in southern Colorado, in July and August of 1979 and 1983. The 1979 studies involved collecting free-flying wasps, separating them by sex and species, and placing their heads, thoraces, and abdomens into separate vials containing pesticide-grade methylene chloride (Burdick and Jackson, Muskegon, Michigan). Since chemicals produced in the head could be smeared onto the abdominal hairs during scent-marking, only males collected from flowers early in the morning before territorial behavior commenced were used in the analysis. Wasps were handled only with forceps.

Male wasps collected in 1983 were dissected to remove their paired mandibular glands and associated reservoirs. Dissected glands were immediately placed in $CH_2Cl_2$. Dissecting tools were rinsed in $CH_2Cl_2$ between each dissection.

Grass stems which had been freshly marked by male *Philanthus* were also sampled in 1983. Our technique consisted of cutting off the part of the stem on

which the male abdomen-dragged and cutting it into sections that fit into a vial containing $CH_2Cl_2$. All stem sections were placed in the vials within 15 sec of being scent marked. The stems were soaked for ca. 2 min with occasional agitation, then the stems were discarded and the extract saved for chemical analysis. Stems were handled only with forceps and scissors. Unmarked control grass stems were collected just outside of the territorial areas of the wasps, and were processed identically to the marked stems.

*Chemical.* All extracts were analyzed by gas chromatography (GC) and mass spectrometry (MS). Initial studies utilized a Perkin-Elmer model 900 gas chromatograph equipped with dual flame ionization detectors and dual 2 m × 3 mm OD glass columns packed with 10% SP-2100 + 3% OV-1 on 100–120 mesh Supelcoport. Oven temperature was 220° isothermal and peak integrations were achieved using a Varian CDS III integrator. Subsequent GC studies were conducted with either a Shimadzu GC-6AM gas chromatograph equipped with dual flame ionization detectors and dual 1.83 m × 3 mm ID stainless-steel columns packed with 3% SP-2100 on 100–120 mesh Supelcoport, or a Hewlett-Packard 5710 gas chromatograph equipped with dual flame ionization detectors and dual 1.83 m × 2 mm ID glass columns packed with 3% OV-101 on Chromosorb W-HP. All GC analyses on either the Shimadzu or Hewlett-Packard GC utilized temperature programing from 100 to 280° at 8°/min.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5710A GC-5982A mass spectrometer interfaced to a Hewlett-Packard 5933 data system. Electron impact (EI-MS) spectra were obtained at 70 eV. Chemical ionization (CI-MS) mass spectra were obtained using ultrapure methane (Airco, Inc.) at a flow rate of 13 ml/min as both carrier and ionizing gas, generating an internal ion source pressure of 0.5 torr. CI spectra were obtained at 200 eV.

The identities of the ketones and aldehydes were facilitated by the formation of the *N,N*-dimethylhydrazones using 1,1-dimethylhydrazine. The addition of this reagent also formed methyl esters of the fatty acids (McDaniel and Howard, 1985). Separation and identification were then made using GC-MS in both EI and CI modes.

Estimates of quantities of 2-pentadecanone present in individual males of *P. basilaris* and *P. bicinctus* were obtained by GC comparisons to a known standard solution of 2-pentadecanone.

## RESULTS AND DISCUSSION

A complex mixture of species-specific volatiles was found in the heads of males of both *P. basilaris* and *P. bicinctus* (Table 1). No volatiles were detected in male abdominal or thoracic extracts, nor in extracts of any body parts of the females. GC and GC-MS analysis of the dissected male mandibular glands con-

TABLE 1. MANDIBULAR GLAND COMPONENTS AND THEIR RELATIVE ABUNDANCES IN MALES OF *Philanthus basilaris* AND *P. bicinctus*[a]

| Component | *P. basilaris* Relative abundance (%, Range)[b] | *P. bicinctus* Relative abundance (%, Range)[c] |
|---|---|---|
| 2-Tridecanone | 0.4 | 0–0.1 |
| 2-Pentadecanone | 53–54 | 24–53 |
| 2-Heptadecanone | 0.2–0.4 | 0–3 |
| $\Delta^x$-Hexadecenoic acid (as methyl ester) | 26–27 | 16–29 |
| Hexadecanoic acid (as methyl ester) | 5–7 | 1–15 |
| $\Delta^{x,y}$-Octadecadienoic acid + $\Delta^x$-octadecanoic acid (as methyl esters) | 7–10 | 3–28 |
| Octadecanoic acid (as methyl ester) | 3 | 1–7 |
| $\Delta^x$-Heptadecenal | 0.1–0.3 | |
| $\Delta^x$-Octadecenal | 2–3 | |
| Ethyl $\Delta^x$-hexadecenoate | | 10–33 |
| Ethyl hexadecanoate | | 1–11 |
| $\Delta^x$-Nonadecenal | | 0.2–7 |
| Ethyl $\Delta^{x,y}$-octadecadienoate + ethyl $\Delta^x$-octadecenoate | | 3–14 |
| Ethyl octadecanoate | | 1–9 |

[a]Symbols $\Delta^x$ and $\Delta^{x,y}$ indicate positions of double bonds not certain.
[b]Ranges based on analyses of two samples of pooled glands.
[c]Ranges based on analyses of four samples of pooled glands.

firmed that these glands were the source of all volatile components initially detected in the male total head extracts. Methylene chloride extracts of grass stems which had been freshly marked by abdomen-dragging territorial males contained all of the volatile components found in the glands of males. Extracts of nonmarked grass stems contained none of the *Philanthus* volatiles.

Ketones and aldehydes in the glands of *P. basilaris* and *P. bicinctus* were easily identified by GC-MS as their corresponding *N,N*-dimethylhydrazones. There was a significant shift in the retention time from that of the parent compounds. The aldehydes had the $m/z = 86$ ion as their base peaks, while the 2-ketones had the $m/z = 100$ ion as their base peaks. These ions are the McLafferty rearrangement ions associated with the hydrazone derivatives and allow unambiguous assignment of the carbonyl location (McDaniel and Howard, 1985). Furthermore, the molecular ions of the hydrazones were such that chemical ionization was not actually necessary.

Mass spectrometry of methyl esters of the fatty acids from the mandibular glands formed by the reagent above is well established as is the mass spectrometry of the ethyl esters associated with *P. bicinctus* (Budzikiewicz et al., 1967). The McLafferty ions at $m/z = 74$ and $m/z = 88$ are characteristic of methyl and ethyl esters, respectively. The molecular weights have been established using CI mass spectrometry.

The double-bond positions of the unsaturated compounds are being determined using methoxy mercuration–demercuration (Abley et al., 1970) but are not yet complete.

The mandibular gland components of *P. basilaris* and *P. bicinctus* show both similarities and differences (Table 1). Both species contain 2-ketones, aldehydes, and fatty acids. Also for both species, 2-pentadecanone is the major constituent. *P. bicinctus* is differentiated from *P. basilaris,* however, by the presence of ethyl esters of the corresponding fatty acids and by the presence of different aldehydes.

The major component of the volatile secretion of both species of *Philanthus* is 2-pentadecanone. This compound has been reported as a frequent component of exocrine secretions, i.e., in the defensive frontal glands of soldier termites of *Schedorhinotermes putorius* (Quennedey et al., 1973), in the mandibular glands of various species of stingless bees in the genus *Trigona* (Blum, 1981), and in the Dufour's glands of formicine ants, including *Lasius, Acanthomyops* (Bergström and Löfqvist, 1970, Wilson and Regnier, 1971), *Camponotus* (Bergström and Löfqvist, 1971), and *Gigantiops* (Blum et al., 1983). However, in the above species, 2-pentadecanone is generally a minor exocrine product.

Although 2-pentadecanone is the major component of the marking volatiles of both *P. basilaris* and *P. bicinctus,* it occurs in different proportions and each species produces several chemicals not used by the other. This species specificity may be important because of the considerable degree of temporal and spatial overlap in activity patterns of *P. bicinctus, P. basilaris,* and other species such as *P. psyche.*

The scent-marking behavior of male *Philanthus* varies with wind speed and direction (Borg-Karlson and Tengö, 1980; O'Neill 1979, 1981). Frequent aggressive interaction between territorial males and conspecific males in *Philanthus* indicates that scent marking does not function to repel intruders; rather, conspecific males who would benefit from usurping a scent-marked territory may be attracted (Alcock, 1975; O'Neill, 1983). Females have also been observed flying upwind toward a scent-marking territorial male just prior to the initiation of copulation (O'Neill, 1979, 1981).

In our study area the territories of *P. basilaris* and *P. bicinctus* frequently intermingled. Territory perches of males of one species were sometimes within several meters of those of the other species and some perches were actually occupied on different days by males of each species. The situation was further

complicated by the fact that both species were active from ca. 1100 to 1400 hr, and a third scent-marking species *P. psyche* also occurred in the same areas at the same time. These biological observations agree with the chemical findings that each species possesses, and benefits by possession of, species-specific blends of pheromonal components.

The results provide direct evidence for marking volatiles in *Philanthus basilaris* and *P. bicinctus*. Not unexpectedly, based on available behavioral and morphological information, heads of male *Philanthus basilaris* and *P. bicinctus* contain significant amounts of volatiles, while male abdomens and female heads and abdomens do not. The hypertrophied mandibular glands are the source of the volatiles, and the quantities present are rather large for wasps of this size.

The volatiles found in the mandibular glands of males of *P. basilaris* and *P. bicinctus* differ in several respects from those of other aculeate wasps. In both males and females of eight species of sphecid wasps (Borg-Karlson and Tengö, 1980; Duffield et al., 1981) and four species of eumenid wasps (Hefetz and Batra, 1980) that have been examined, pyrazines were the only volatile componds detected in head extracts. In *Philanthus triangulum,* in which males are territorial and exhibit abdomen-dragging, not only did both sexes produce pyrazines, but females had greater amounts than males (Borg-Karlson and Tengö, 1980). Males of the two *Philanthus* we studied produced much greater amounts of volatiles per individual than did males of *P. triangulum* (*P. bicinctus:* ca. 240 μg/head; *P. basilaris:* ca. 220 μg/head; *P. triangulum:* 0.05 μg/head). This cannot be attributed to differences in size among the species, since *P. triangulum* is only slightly smaller than *P. basilaris.* The exact biological role of the volatiles reported in this paper are not known, although behavioral evidence suggests that they act as components of marking, attracting, and sex pheromones.

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# USE OF PREDATOR ODORS AS REPELLENTS TO REDUCE FEEDING DAMAGE BY HERBIVORES
## I. Snowshoe Hares (*Lepus americanus*)

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**Abstract**—The effectiveness of predator odors (fecal, urine, and anal scent gland) in suppressing feeding damage by snowshoe hares was investigated in pen bioassays at the University of British Columbia Research Forest, Maple Ridge, British Columbia, Canada. A total of 28 bioassay trials tested the effects of these odors on hare consumption of willow browse and coniferous seedlings. Lynx and bobcat feces, weasel anal gland secretion, and lynx, bobcat, wolf, coyote, fox, and wolverine urines resulted in the most effective suppression of hare feeding damage. Novel odors of domestic dog urine and 2-methylbutyric acid did not reduce feeding. A field bioassay with lodgepole pine seedlings and weasel scent provided significant results comparable to the pen bioassays. The short-term (up to seven days) effectiveness of these treatments was more likely due to evaporative loss of the active repellent components of a given odor than habituation of hares to the stimulus. Predator odors as repellents have a biological basis compared with the anthropomorphic origins of commercial repellents. When encapsulated in weather-proof controlled-release devices, these odors could provide long-term protection for forestry plantations and agricultural crops which experience hare/rabbit feeding damage.

**Key Words**—Bioassays, conifer seedlings, crop protection, feeding activity, herbivores, *Lepus americanus*, predator odors, repellent, snowshoe hare.

## INTRODUCTION

Chemical communication plays a vital role in the lives of many mammalian species. Most research, to date, has been concerned with the origins and diversity of pheromones and their effects on behavior and reproduction within a given



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species (Bronson, 1971; Chael and Sprott, 1971; Ralls, 1971; Eisenberg and Kleiman, 1972; Stoddart, 1980a; Müller-Schwarze, 1983). However, interspecific signals or allelochemics (Whittaker and Feeny, 1971) are also biologically meaningful in terms of survival and other communicative attributes. This is particularly important with respect to the detection of predators by prey.

In general, recognition of a predator through the perception of olfactory cues is a widespread phenomenon among mammals (see Stoddart, 1980a). Experimental evidence which demonstrates the influence of predator odors on prey behavior includes reduced feeding by black-tailed deer (*Odocoileus hemionus*) when exposed to fecal odors (Müller-Schwarze 1972), distributional patterns of white-tailed deer (*O. virginianus*) closely correlated with the boundaries of wolf pack territories (Mech, 1977; Rogers et al., 1980), behavior of ground squirrels (*Spermophilus beecheyi*) to predatory snake odor (Henessy and Owings 1978), and reduced capture of short-tailed voles (*Microtus agrestis*) exposed to weasel odor (Stoddart, 1976, 1980b, 1982). A study by Boonstra et al. (1982) analyzed the effect of shrew odor on depressing trappability of voles (*M. pennsylvanicus*). The importance of predator odor on the vigilance states of the rat has been discussed by Cattarelli and Chanel (1979) and Vernet-Maury et al. (1984).

Thus, the odors (fecal, urine, scent gland) of predators appear to elicit a "fear response" when detected by prey species. This phenomenon could be of great value as a repellent system to suppress feeding by herbivores on forest and agricultural crops. The search for applications of chemical signals (attractants and repellents) in wildlife management and crop protection has been discussed by Christiansen and Doving (1975), Shumake (1977), and Stoddart (1980a). In this regard, one of the most important herbivores which affects coniferous regeneration in the boreal forests of North America is the snowshoe hare (*Lepus americanus*). These hares reach a peak in abundance every 9–10 years and can cause serious damage by feeding on commercial forestry plantations (Sullivan and Sullivan, 1982; Sullivan, 1984).

In general, standard repellents have been ineffective in suppressing feeding by herbivores such as the snowshoe hare. This is not surprising because the majority (if not all) of commercial animal repellents were developed because of their distasteful odor to man rather than to the problem animal. This makes the unlikely assumption that herbivores have the same olfactory limits and preferences as man. A repellent should elicit fear, not simply "displeasure," such as would be the result of a repugnant chemical (Lichtenstein, 1950; Seward and Raskin, 1960; Lehner et al., 1976). An effective repellent should result in an immediate and long-lasting avoidance of the source of that odor (Solomon and Wynne, 1953).

This study was designed to test the hypothesis that predator odors will act as biological repellents, based on a "fear response," to suppress snowshoe hare feeding on coniferous tree seedlings. To provide a comprehensive analysis of the effect of predator odors on hare feeding behavior, the full range of terrestrial

predator species in the families Canidae, Felidae, and Mustelidae were investigated. The impact of these odors on snowshoe hares and their potential use as repellents are discussed.

## METHODS AND MATERIALS

*Pen Bioassays.* Bioassays were conducted at the Univesity of British Columbia Research Forest, Maple Ridge, British Columbia, Canada. Duplicate enclosures (one control and one treatment) (6 × 18 m) in a natural environment (all native vegetation was removed prior to start of trials) served as bioassay pens (Figure 1). Within each pen, four covered experimental units (2 × 3 × 1 m) shielded the test material from precipitation, while allowing hares free access to feed on material under them. Hares moved freely between each pen by way of openings in the adjoining fence. To exert adequate feeding pressure on the experimental material, three to five hares were used in each trial. All hares used (total of 52) were from our northern study areas at Prince George, British Co-

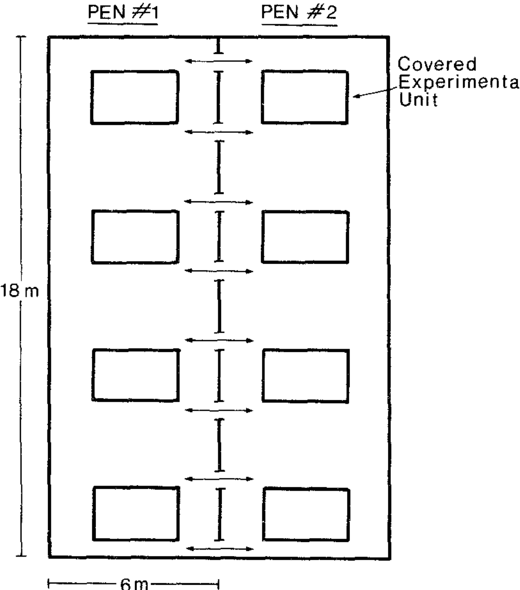


FIG. 1. Duplicate enclosures used in the predator odor bioassay trials. Each of the four units within a pen consisted of 10 replications of control or treatment material. A replicate was 10 twigs or seedlings vertically anchored in a styrofoam block. Ten such blocks were systematically located within each unit.

lumbia. Hares were replaced at regular intervals to overcome potential variability and habituation among animals. A total of 28 bioassay trials were conducted from October 1981 to March 1982. Rabbit pellets and water were available *ad libitum* throughout the study.

The effectiveness of predator odors (feces, urine, and anal scent gland preparations) as biological repellents were conducted in standard pen bioassay tests. Feces tested included lynx (*Lynx canadensis*), bobcat (*L. rufus*), cougar (*Felis concolor*), coyote (*Canis latrans*), wolf (*C. lupus*), and wolverine (*Gulo gulo*). Urine tested included lynx, bobcat, coyote, wolf, fox (*Vulpes vulpes*), and wolverine. Anal gland preparations tested were weasel (*Mustela erminea*), marten (*Martes americana*), fisher (*Martes pennanti*), striped skunk (*Mephitis mephitis*), mink (*Mustela vison*), and wolverine. Fecal samples were collected from the Okanagan Game Farm, Penticton, British Columbia; Vancouver Game Farm, Aldergrove, British Columbia; and the Olympic Game Farm, Sequim, Washington. Urine samples were from the Triple D Game Farm, Kalispell, Montana. Anal gland samples (weasel and wolverine) were collected from fur trappers at Prince George, British Columbia, and kept frozen prior to use. These glands were dissected and their contents used in the bioassays. Macerated anal glands of marten and fisher and anal gland secretions of skunk and mink were commercially available.

Body odors (bedding, body scent, and pen) of a captive live coyote were also tested. The bedding consisted of alfalfa hay in which the coyote had urinated, defecated, lain, and eaten for a 2-week period. This bedding was spread around the perimeter of an experimental unit prior to testing. In a second experiment, two towels were dampened with water and rubbed on the back and sides of the coyote on three consecutive days. The towels were cut into strips with one strip placed in the center of a given styrofoam block and then surrounded by the willow test material. A third experiment was done in the enclosed coyote pen (3 × 15 m) and an adjoining control pen which had never been used by the animal. The coyote was removed for the experiment to assess the impact of its pen odor on hare feeding. One hundred willow twigs were placed in a 30 × 90 cm styrofoam block in each of the two adjacent pens. The procedure followed for these experiments was identical to that described in the next section on experimental design. Two additional experiments determined if hare feeding would be depressed by any novel odor. Domestic dog urine was tested against wild coyote urine and a control. The second test utilized 2-methylbutyric acid, a very unpleasant (stench) odor compared with a control. Again, the procedure followed was identical to that outlined in the next section.

*Experimental Design.* Each of the four units within the control and treatment pens consisted of 10 replications of test material. For a given treatment, all 10 replicates within a unit were identical. Whenever possible (depending on supply of test material), all four units in the treatment pen contained the same predator odor preparation. When each of the units within the treatment pen had

different preparations, they were compared with the corresponding units in the control pen. A replicate was 10 twigs vertically anchored in a styrofoam block (30 × 30 × 3 cm). Ten such blocks were systematically located within each unit. This pattern of twigs simulated a natural distribution of available browse.

The size of browse items was standardized to stems 1.0 cm in diameter or less and heights of 30 cm or less. Browse damage (twig length consumed) was surveyed at daily intervals during each bioassay until feeding activity was at a very low level. Percentage of twig lengths consumed indicated actual utilization of a given browse treatment relative to available browse. Willow (*Salix* spp.) browse, a preferred food of snowshoe hares, was used to evaluate candidate predator odors during the initial part of the study. The most effective odors were then tested on 1-year-old nursery lodgepole pine (*Pinus contorta*) and white spruce (*Picea glauca*) seedlings. Variation in seedlings was minimal, as similar seedlots were supplied by the British Columbia Ministry of Forests for all trials.

Predator odor preparations (10 ml or g) were dispensed in liquid form in Petri plates (100 × 15 mm) with one plate per styrofoam block. A comparable amount of water was placed in Petri plates in control blocks. Concentrations were maximized as much as possible (4 : 1 ratio of feces with water; urine and scent gland material without dilution) for best performance. The covered experimental units not only protected the bioassays from adverse weather conditions, but also helped to keep the odor around the browse items for maximum evaluation purposes. Thus, concentration of an odor was controlled to some degree but evaporation rate was not.

This design and our analysis of variance technique follow the randomized complete block design and analysis described by Dodge et al. (1967) for forest-mammal repellent tests. Percentage data for twig length browsed were transformed by arcsin square root prior to analysis. Duncan's multiple-range test was then conducted to determine significantly different browse treatments.

*Field Bioassay.* A field test of weasel scent gland odor, a novel odor (isoamyl methyl sulfide), and a control was conducted at Tabor Mountain, 25 km southeast of Prince George, British Columbia, during October 10–17, 1982. This study area was located in the subboreal spruce biogeoclimatic zone (Krajina, 1969). It was overgrown with deciduous brush species, mainly willow (*Salix* spp.) and Sitka alder (*Alnus sinuata*), with some aspen (*Populus tremuloides*), black cottonwood (*P. trichocarpa*), and paper birch (*Betula papyrifera*), and had experienced coniferous plantation failure because of browsing damage by snowshoe hares. Population density of hares in this area in October 1982 was approximately 8 per hectare (Sullivan and Sullivan, unpublished) which is near peak densities of this species (Keith and Windberg, 1978).

Three blocks (each 400 $m^2$) of 50 (5 × 10) 1-year-old lodgepole pine seedlings each were planted on October 10, 1982. Blocks were assigned treatments as follows: control, weasel anal gland odor, and isoamyl methyl sulfide (stench). Blocks were separated by 100 m to avoid confounding of odors among treat-

ments. A 5-ml plastic scintillation vial was attached by a plastic twist-tie to each seedling and filled with 2.5 ml of the appropriate test material. Vials were also attached to control seedlings. These vials helped protect the compounds from adverse weather conditions and kept the odor around the seedlings for optimum evaluation purposes. Browsing of seedlings was monitored daily for the first four days and finally on day 7 of the experiment. Any feeding (clipping) on the terminal shoot was considered as mortality to a given seedling. Seedlings rarely recover from terminal feeding damage and even if they do, growth and form of the young tree are usually severely impaired.

## RESULTS

*Predator Fecal Odors.* The effectiveness of predator fecal odors in suppressing hare browsing of willow twigs is illustrated in Figure 2. Bobcat and

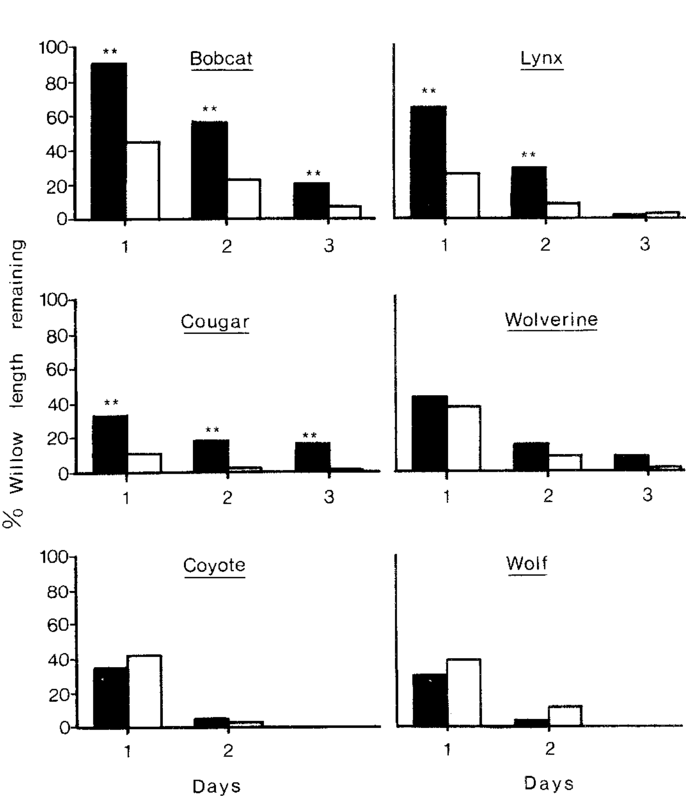


FIG. 2. Effectiveness of predator fecal odors in suppressing snowshoe hare browsing of willow twigs (**$P < 0.01$, analysis of variance). Fecal odor treatment represented by shaded and control by unshaded histograms.

lynx feces consistently suppressed hare feeding with a highly significant ($P <$ 0.01) difference between controls and treatments. Hares did reduce the quantity of treated material by approximately 35% each day, but still consumed significantly more control willow than that protected by the bobcat and lynx feces. Cougar feces also significantly reduced feeding compared with a control but at a much less (one-third) effective level than the bobcat. Fecal odors from the coyote, wolf, and wolverine were not effective in suppressing willow feeding by hares.

*Predator Urine Odors.* The effectiveness of predator urine in reducing hare feeding is shown in Figure 3. Urine of all species significantly ($P < 0.01$) lowered browsing compared with controls. Again, hares did feed on the treated material during each experiment but they always ate significantly less than in the control units.

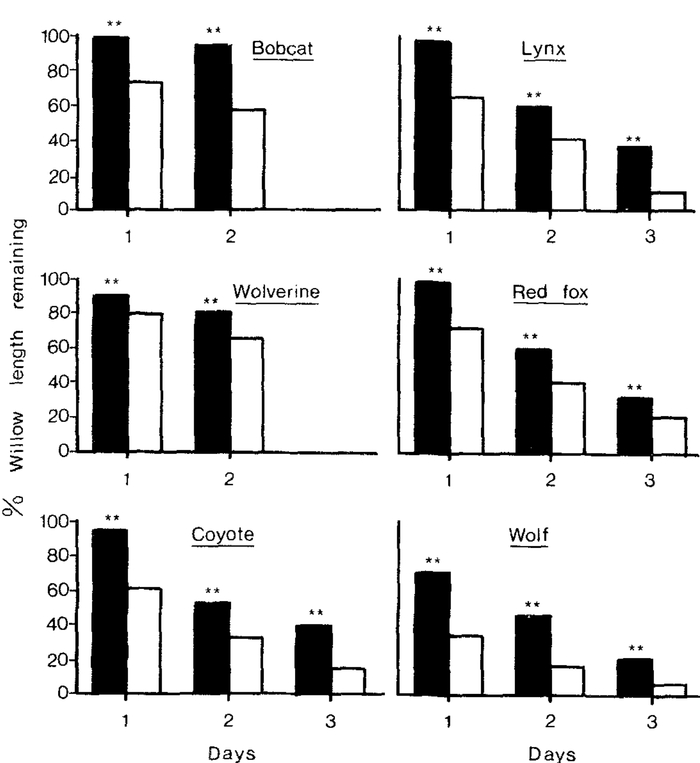


FIG. 3. Effectiveness of predator urine odors in suppressing snowshoe hare browsing of willow twigs ($^{**}P < 0.01$, analysis of variance). Urine odor treatment represented by shaded and control by unshaded histograms.

*Predator Scent Gland Odors.* All scent gland extracts tested were from the family Mustelidae (Figure 4). In all species, except the wolverine, scent gland odor suppressed hare feeding with significant ($P < 0.01$) differences between treated material and controls. Weasel scent was the most effective odor among the mustelids for reduction of hare browsing. Wolverine scent was effective ($0.01 < P < 0.05$) during the first day but declined thereafter. Only one set of fresh glands was available for the trial, and this small amount of material may explain the somewhat marginal response of hares to this odoriferous species.

*Predator Body and Novel Odors.* The initial responses of hare feeding to the body odors of a live predator, the coyote, are illustrated in Figure 5A. In

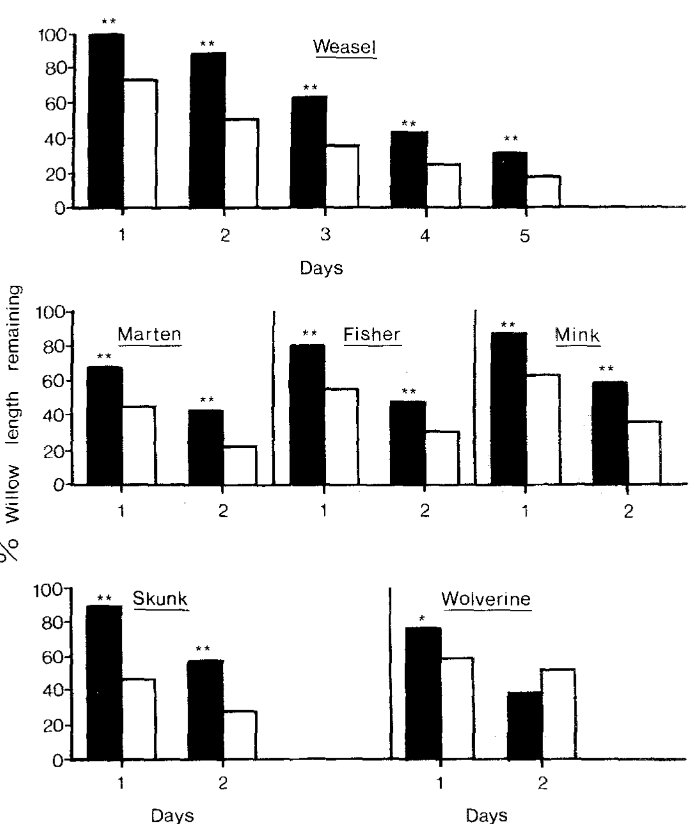


FIG. 4. Effectiveness of predator scent gland odors (Mustelidae) in suppressing snowshoe hare browsing of willow twigs (* $P < 0.05$; ** $P < 0.01$, analysis of variance). Scent gland odor treatment represented by shaded and control by unshaded histograms.

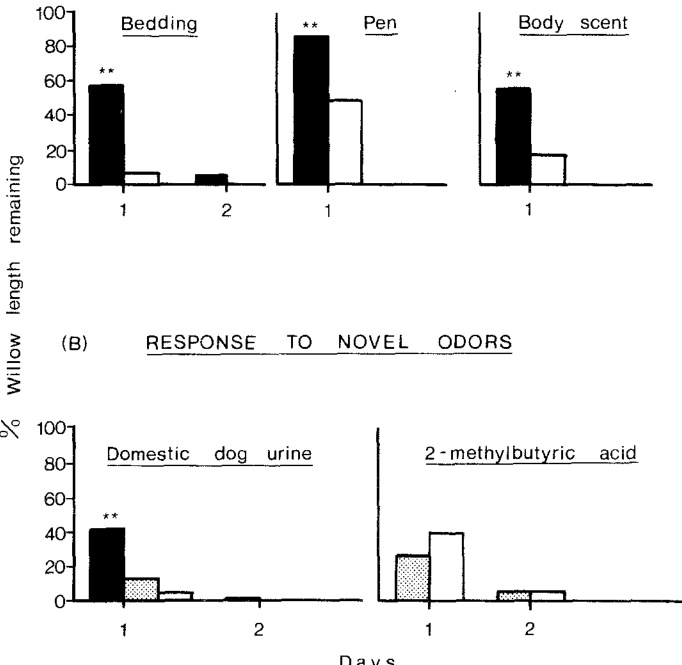


FIG. 5. Effectiveness of (A) live coyote body odor and (B) novel odors in suppressing snowshoe hare browsing of willow twigs (** $P < 0.01$, analysis of variance). Coyote body odor treatments represented by shaded and control by unshaded histograms. Coyote urine ■, domestic dog urine and 2-methylbutyric acid ▩, and control □.

all cases, hares reacted negatively to the odors by consuming significantly less willow than in adjacent controls.

To determine if hares were responding to the predator odors in this study because of the novelty of the smell, trials with domestic dog urine and 2-methylbutyric acid were conducted. The results are shown in Figure 5B. The domestic dog urine did not affect hare feeding behavior when compared with a control. In addition, this dog urine acting as a novel odor resulted in significantly ($P < 0.01$) more willow browse consumed by hares than the wild coyote urine treatment. Similarly, the 2-methylbutyric acid (stench) failed to suppress hare feeding relative to a control.

*Predator Odors and Conifer Seedlings.* The most effective predator odors from the willow feeding trials were then tested on lodgepole pine and white

spruce seedlings. In general, snowshoe hares prefer pine seedlings over spruce, and this pattern was consistent throughout our trials. The bobcat fecal odor formulation significantly suppressed hare feeding on both pine and spruce seedlings compared with the controls which were almost entirely eaten after one day (Figure 6). After the initial (first day) minor feeding on seedlings, replacement of treatment with fresh fecal material maintained a consistent response until after the fourth day when seedling consumption increased. Red fox and lynx urines initially suppressed seedling browsing by hares before declining at days 3 and 4, respectively (Figure 7). Again, control seedlings were essentially exhausted after the first day of each urine odor trial.

Results of the pen and field bioassays of weasel scent gland odor are illustrated in Figure 8. In the pen bioassay, hares did not feed on the weasel odor conifer seedlings for the first two days of the experiment while the controls were completely eaten. A similar result was recorded in the field bioassay. In both trials, the suppressing effect of weasel odor on seedling consumption declined after four days, and this occurred whether control seedlings were replaced or not. The novel odor, isoamyl methyl sulfide (stench), used in the field trial was initially (one day) effective and then seedling survival declined to levels less than the control block. This rapid decline was not due to evaporative loss since

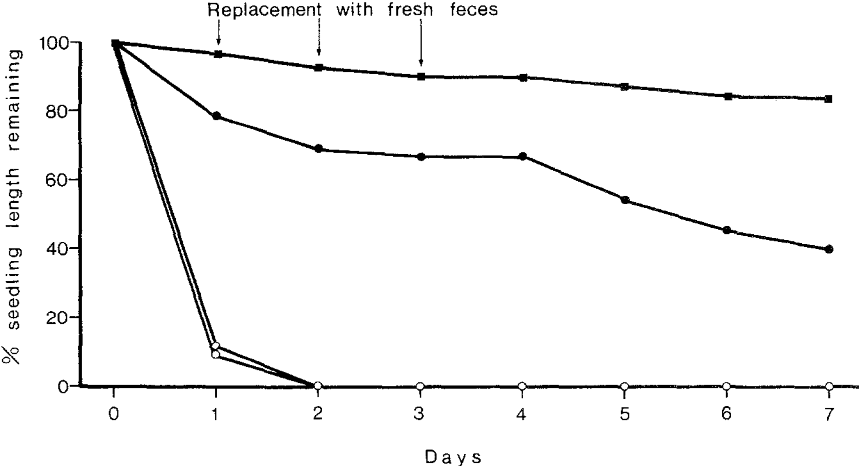


FIG. 6. Suppression of snowshoe hare browsing of white spruce, ■, and lodgepole pine seedlings, •, with bobcat fecal odor. Control seedlings represented by ○. Treatment material was replaced with fresh feces on each of the first three days.

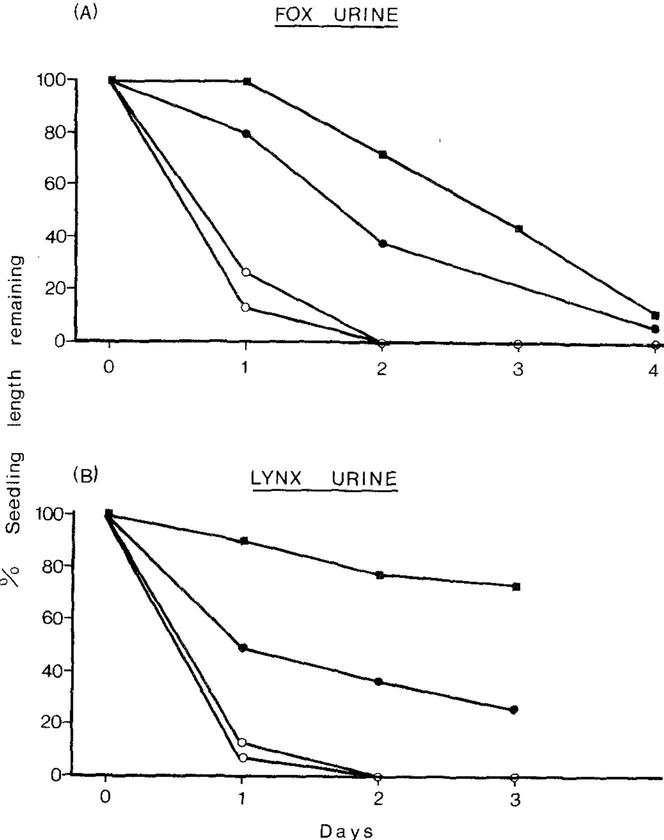


FIG. 7. Suppression of snowshoe hare browsing of white spruce, ■, and lodgepole pine, •, seedlings with (A) red fox urine odor and (B) lynx urine odor. Control seedlings represented by ○.

some compound was still present in the vials at the end of the trial. The effect of marten scent gland odor on seedling consumption is shown in Figure 9. Comparable results to the urine trials (Figure 7) were obtained with control seedlings almost entirely eaten after one day and spruce seedlings surviving better than pine until the fourth day of the trial.

## DISCUSSION

The rationale for our investigation was to screen as many odors of terrestrial predators (order Carnivora) of the snowshoe hare as possible. The distri-

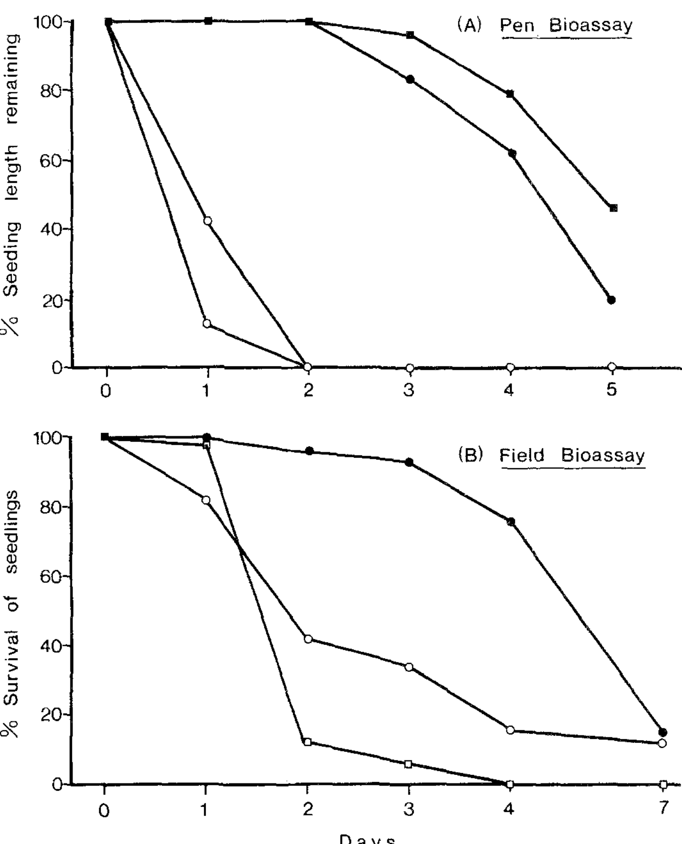


FIG. 8. Suppression of snowshoe hare browsing with weasel scent gland odor in (A) pen bioassay of white spruce, ■, and lodgepole pine, •, seedlings with controls ○; and (B) field bioassay of lodgepole pine seedlings with weasel scent, •, isoamyl methyl sulfide (novel odor) □, and a control ○.

bution of each predator species was, in part, sympatric with that of the snowshoe hare in central British Columbia (Banfield, 1974). Potential sources of odor were fecal (Muller-Schwarze, 1972) which possibly originate from anal sacs (Albone et al., 1974; Albone and Perry, 1976; Preti et al., 1976), urine, and anal scent glands (Stoddart, 1980a).

This study has clearly demonstrated the influence of predator fecal, urine, and scent gland odors in altering the feeding behavior of snowshoe hares. Although only short-term (up to seven days) results were obtained, certain predator

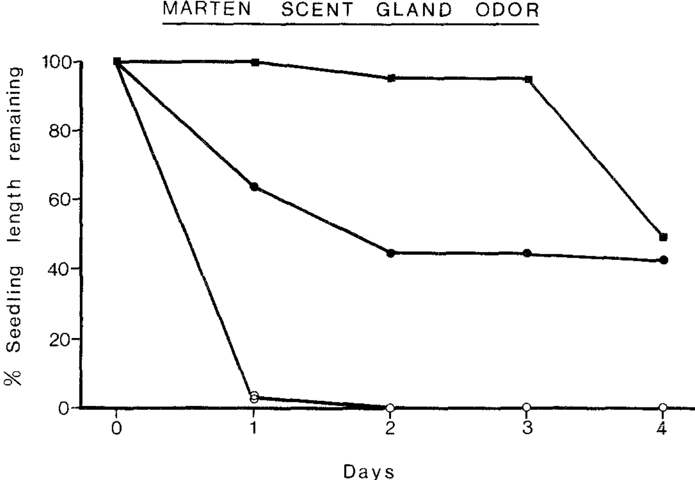


FIG. 9. Suppression of snowshoe hare browsing of white spruce, ■, and lodgepole pine, •, seedlings with marten scent gland odor. Control seedlings represented by ○.

odors significantly suppressed feeding by hares on willow and coniferous seedlings when compared with controls. This suppression was not because of the strangeness or novelty of a given odor, because hares were not suppressed by domestic dog urine, 2-methylbutyric acid, or isoamyl methyl sulfide. They readily consumed seedlings treated with these novel odors. Thus, if we overlook temporal constraints at this stage, it seems plausible that the hypothesis of predator odors eliciting a "fear response" in snowshoe hares, and thereby suppressing their feeding on a given food source, is valid.

The effectiveness of lynx and bobcat feces and urine in altering hare feeding behavior in our bioassay trials is not surprising since populations of the former tend to follow the cyclic fluctuations of snowshoe hares, and hence is a major predator (Brand and Keith, 1979). Similarly, the coyote also heavily utilizes snowshoe hare prey during peak populations in the boreal forest of western Canada (Todd et al., 1981). However, coyote feces and those of the wolf did not deter hares from feeding on willow browse. Since urine odors of all three canid species (coyote, wolf, fox) significantly suppressed feeding, perhaps the anal sac secretions typical of the Canidae were not present in the feces tested. If this was the case, hares might not have identified the odor of a potential predator in the fecal samples. Thus, predator urines may be generally good indicators of the presence of a predator in a given area. Wolf urine had the lowest amount (71.5%) of remaining willow browse after the first night of a trial compared with an average of 95.8% for the other five species (see Figure 7). This is not surprising since wolf and cougar (whose fecal odors were not effective relative to lynx and bobcat) prey mainly on larger mammals than the snowshoe hare.

The general effectiveness of all mustelid scent gland preparations is indicative of this family which readily use anal scent gland secretions for various modes of communication ranging from defense in the skunk to expression of sexual status in the mink (Stoddart, 1980a). Wolverine anal gland secretion was the least effective in our bioassays, possibly owing to lack of a substantial sample. Further work on chemical communication is certainly needed for this mustelid species. The outstanding efficacy of weasel odor for both willow and conifer seedlings was rivaled only by the lynx and bobcat feces in this study. Weasels primarily prey on mice and voles but will attack adult hares and rabbits, and particularly juveniles at the nest site. Lockley (1964) has postulated that rabbits have an innate fear of mustelids because of their ferocity and efficiency as predators.

The occasions for avoidance learning by prey animals are very limited, and presumably the given individual uses its innate species-specific defense reaction whenever it encounters a novel environmental stimulus (Bolles, 1970). As discussed by Stoddart (1980b), this argument makes the usefulness of a novel odor in a given experiment somewhat suspect. However, in terms of our field bioassay, the novel odor was initially rejected but later accepted (see Figure 8), which is the predicted response.

The advantages and disadvantages associated with interpretation of results from laboratory or captivity studies have been discussed by Mech and Peters (1977) and Stoddart (1980b). To date, only Stoddart (1980b, 1982) and Boonstra et al. (1982) have investigated the effects of predator odors on potential prey in the field. Our pen environment was close to a field situation but kept hares at artificially high densities (23 times the density of a peak population) and did not allow them to disperse or "escape" from the experimental set-up or agonistic social interactions among individuals. Both these factors are important because they may help explain why hares did feed on willow twigs and conifer seedlings in the presence of predator odors. If the predicted fear response is real, one would expect there to be little or no hare activity (and hence feeding) in the areas with odor of a predator. However, the high density of animals and frustrated dispersal presumably resulted in hares feeding on the experimental material, but always at a statistically significant lower level than the controls, at least for the effective predator odors.

If this was the case, the question then arises as to why the weasel odor in the field bioassay did not maintain a consistent suppression of hare feeding? This trial was conducted in a natural situation where hares could leave or avoid the area with a dangerous (high risk) predator odor. The most readily available answer is that the hares habituate to the odor, realize the predator is not really present, and start to feed on the given material. There are risks in expecting a repellent to continue working in the absence of further reinforcement, but other authors (Bolles, 1970; Hinde and Stevenson-Hinde, 1973; Muller-Schwarze, 1974) indicate that innate responses to naturally dangerous olfactory signals

should not habituate, nor should they require learning experiences. A study by Bullard and Shumake (1977) indicates that odor and taste stimuli that occur naturally in the ecosystem have much more durable effects than those selected from nonbiological origins.

Before we analyze this important problem any further, it should be stressed that our experimental procedure had very little control of concentration of the active components of a given fecal, urine, or scent gland sample. In addition, we had virtually no control of evaporation rate of the volatile functional compounds in each sample. These restraints are critically important because they may explain the decline in effectiveness of a predator odor over time. Loss of the one or more active compounds (kairomones) (Brown et al., 1970) in a predator odor would terminate this interspecific communication between predator and prey. This assumption was tested by replacing the bobcat feces with fresh samples which maintained a more consistent suppression of hare feeding (see Figure 6). Unfortunately, experimental units with original fecal samples present throughout the trial were not available for comparison. In general, all previous studies of predator odors affecting the behavior of mammalian prey have also been short-term in duration (Müller-Schwarze, 1972; Henessy and Owings, 1978; Cattarelli and Chanel, 1979; Stoddart, 1980b; Boonstra et al., 1982).

If the short-term response to our odor samples was due to evaporative loss of the active repellent components rather than habituation by hares, then identification, synthesis, and testing of one or more of these compounds should suppress browsing damage for a considerable period. This hypothesis is presently being tested, and to date, a component of weasel scent (Crump, 1978, 1980) has successfully suppressed feeding damage to seedlings by hares for a six-week period in a field bioassay trial (Sullivan and Crump, 1984). This period covered the early spring when conifer seedlings were susceptible to hare damage, just after snow-melt and prior to hares switching to preferred summer herbaceous foods. This compound would presumably have maintained protection for seedlings if hares had continued to feed on coniferous material. Further work with other mustelid scents, lynx–bobcat feces, and some of the predator urines is in progress.

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# USE OF PREDATOR ODORS AS REPELLENTS TO REDUCE FEEDING DAMAGE BY HERBIVORES

## II. Black-Tailed Deer (*Odocoileus hemionus columbianus*)

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**Abstract**—The effectiveness of predator odors (fecal and urine) in suppressing feeding damage by black-tailed deer was investigated in pen bioassays at the University of British Columbia Research Forest, Maple Ridge, British Columbia, Canada. A total of eight bioassay trials tested the effects of these odors on deer consumption of salal leaves and coniferous seedlings. Cougar, coyote,and wolf feces as well as coyote, wolf, fox, wolverine, lynx, and bobcat urines provided the most effective suppression of deer feeding damage. Novel odors of ammonia and human urine did not reduce feeding. Predator fecal odor formulations in direct foliar application, adhesive application, and in plastic vials were all effective in suppressing deer feeding. Of all urines tested, coyote provided the most consistent suppression of deer browsing on salal. Deer consumed significantly more untreated Douglas fir and western red cedar seedlings than those protected by coyote urine odor. The active repellent components of predator odors which suppress deer feeding may be suitable for encapsulation in controlled-release devices which could provide long-term protection for forest and agricultural crops.

**Key Words**—Herbivores, feeding damage, black-tailed deer, *Odocoileus hemionus columbianus*, repellents, predator odors, feces, urine, scent gland compounds, forest and agricultural crops.

## INTRODUCTION

The detection of predators by prey species is a vitally important function of chemical communication among mammals. Several studies outlined by Sullivan



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et al. (1984) indicate the effect of predator odors on prey behavior, particularly feeding by herbivores. This interspecific relationship has also been observed among prey species of the family Cervidae and their predators. White-tailed deer (*Odocoileus virginianus*) in the wild avoid areas of maximum wolf activity (Hoskinson and Mech, 1976; Mech, 1977; Rogers et al., 1980). This avoidance could be in response to fecal, urine, or scent gland odors. In a more controlled environment, predator fecal odors have resulted in significant suppression of feeding activities of black-tailed deer (*O. hemionus columbianus*) (Müller-Schwarze, 1972, 1983; Melchiors and Leslie, 1984).

Feeding damage by cervids to commercial coniferous and deciduous forest regeneration and tree fruit production (Anthony and Fisher, 1977) is common throughout the distribution of members of this herbivore family.The reforestation problem is particularly severe in North America (Crouch, 1976; Black et al., 1979; Brodie et al., 1979) but also occurs in Europe (Dzieciolowski and Szukiel, 1976; Giban, 1976; Myllymaki, 1976) and parts of Asia (Ghosh, 1976). Browsing by deer suppresses height growth and productivity and may cause tree mortality. As discussed by Sullivan et al. (1984), standard repellents have generally not been effective in preventing browsing by herbivores such as deer.

Studies have shown that olfaction is an important part of food selection by deer (Longhurst et al., 1968; Schwartz et al., 1980). This suggests that an olfactory repellent could prevent any damage from occurring while a taste repellent would require damage to occur before it could be detected and elicit the desired behavior. This study was designed to test the hypothesis that olfactory repellency based on a "fear response" from one or more predator odors will suppress feeding by deer on coniferous tree seedlings. The impact of predator odors on black-tailed deer and their potential development as repellents is discussed.

## METHODS AND MATERIALS

*Pen bioassays.* Bioassays were conducted at the University of British Columbia Research Forest, Maple Ridge, British Columbia, Canada. An enclosure (0.6 hectare) in a natural environment (all native vegetation was removed prior to start of trials), adjacent to a stand of coastal coniferous forest, served as a bioassay pen. To exert sufficient feeding pressure on the experimental material, four to seven adult black-tailed deer were used in each trial. All deer used were from the resident herd maintained by the University Research Forest. Two bioassay trials were conducted during July to August 1980, four trials during May to August 1981, and two trials from March to April 1982. Commercial deer chow and water were available ad libitum throughout the study.

The effectiveness of predator fecal and urine odors as biological repellents to suppress feeding by deer were conducted in standard pen bioassay tests (Gau-

ditz, 1977). Feces tested included cougar (*Felis concolor*), coyote (*Canis latrans*), wolf (*C. lupus*), combined lynx (*Lynx canadensis*)-bobcat (*L. rufus*), and jaguar (*Panthera onca*). Urine tested included coyote, wolf, lynx, bobcat, fox (*Vulpes vulpes*), and wolverine (*Gulo gulo*). Fecal samples were collected from the Okanagan Game Farm, Penticton, British Columbia, and Vancouver Game Farm, Aldergrove, British Columbia. Urine samples were from the Triple D Game Farm, Kalispell, Montana. Several additional experimental compounds (human urine and ammonia) were tested as novel odors. In the initial two trials in 1980 and one trial in 1981, commercial repellents (No. 1, based on extracts of hot pepper; No. 2, based on putrescent eggs) were tested with the predator odors.

*Experimental Design.* The basic design of a bioassay trial in this study utilized one or two completely randomized plots (blocks) with 10–20 replications of each of several treatments and an untreated control. A replicate consisted of one salal (*Gaultheria shallon*) branch with 10 leaves. Salal branches were stapled to evenly spaced (6-m intervals) wooden stakes (5 × 55 × 122 cm) anchored in the ground. Salal was used because the leaves remain palatable for the duration of pen bioassay. They are also a preferred food of black-tailed deer in a pen situation.

Browse (feeding) damage was surveyed daily during each bioassay until feeding activity at the test sites was at very low levels. For each salal branch, the number of leaves consumed, torn off, or bitten was recorded as browsed. The average percentage of a treatment browsed was then calculated from the number of leaves damaged. The final trial used 2-year-old nursery western redcedar (*Thuja plicata*) and Douglas fir (*Pseudotsuga menziesii*) seedlings. Percentage of seedling length consumed indicated actual utilization of a given seedling browse treatment relative to available browse. A replicate consisted of two seedlings whose lengths had been measured prior to the experiment.

In trials 1, 2, and 3, feces of each predator species were mixed (4:1 ratio) with water in a Waring blender to the consistency of a slurry. The slurry was then filtered through cheesecloth and the filtrate collected. Salal branches and leaves were dipped in the filtrate to the point of runoff and then allowed to dry. A comparison of the fecal filtrate and the residue (bone, hair) left after filtration was conducted in trial 3. The commercial repellents were prepared according to label instructions and applied to the salal leaves in an identical manner. Control salal remained untreated.

In trial 4, the novel odors of ammonia and human urine were dispensed in 15-ml plastic scintillation vials which were attached to a respective stake directly below the salal leaves. In this and all subsequent trials using vial application, empty vials were also attached to stakes with control salal leaves. A 7.5 × 7.5-cm piece of sheet metal was attached to the top of each stake to help protect the vials from rain.

In trial 5, fecal extracts were mixed with Rhoplex AC-388 adhesive and

this liquid mixture was painted on a given stake directly underneath the salal leaves. The adhesive by itself was applied to stakes with control salal. Trial 6 compared adhesive and vial applications of fecal material with the preparatory procedure identical to that described previously. Vials and adhesive mixtures were positioned on a given stake to keep an odor around the salal leaves for optimum evaluation purposes. Again, as described in Sullivan et al. (1984), concentration of an odor and exposure to adverse weather conditions were controlled to some degree but evaporation rate was not.

In trial 7, predator urines (undiluted) were dispensed in vials with three predator species and one control in each of two plots. This trial was conducted for six days, and vials were then removed from all treated salal. Feeding damage was then surveyed for a seventh day. Trial 8 assessed the effectiveness of coyote urine in suppressing deer feeding on planted cedar and fir seedlings. Two vials were placed adjacent to the base of each fir and one vial next to the base of each cedar seedling. This trial was replicated in two identical plots with five replications of control and treated cedar and fir in each plot.

The statistical analysis of variance and experimental design correspond with those outlined by Dodge et al. (1967) for forest-mammal repellent tests. Percentage data for seedling length browsed were transformed by arcsin square root prior to analysis.

## RESULTS

*Predator Fecal Odors—Foliar Application.* The effectiveness of predator fecal odors in suppressing deer browsing of salal leaves is illustrated in Figure 1. Cougar, coyote, and wolf feces completely suppressed feeding during the 20-day trial. The commercial repellent No. 2 was also as effective as these feces. However, commercial repellent No. 1 declined to a level comparable with the control after nine days. Both repellent No. 1 and control salal leaves were essentially exhausted at the completion of the trial.

The above trial was replicated with the addition of lynx–bobcat feces (Figure 2). Again, cougar fecal odor completely suppressed feeding by deer. The other predator odors of coyote, wolf, and lynx–bobcat were also very effective, with an average of 93.2% salal leaves remaining at the completion of the trial. Commercial repellent No. 2 was as effective as the fecal odors with 94.5% salal left after 18 days. Repellent No. 1 and the control leaves were readily eaten and declined to levels of 21.5% and 13.0%, respectively.

To further evaluate the efficacy of predator fecal odors in adverse weather conditions (heavy rain), a third trial with coyote, jaguar, and wolf feces compared with commercial repellent No. 2 and a control was conducted (Figure 3). During the first three days of the trial, significantly ($P < 0.01$) more control salal was eaten compared with all treatments. However, after 24 hr of heavy

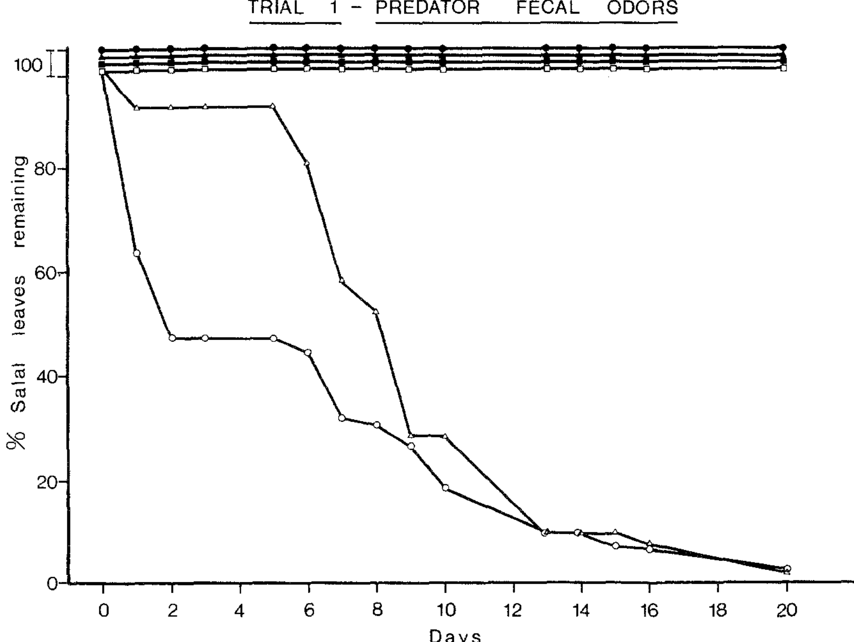


FIG. 1. Effectiveness of predator fecal odors in suppressing deer browsing of salal leaves. Treatments are cougar ●, coyote ▲, wolf ■, commercial repellent No. 1 △, commercial repellent No. 2 □, and control ○.

rain, the fecal and repellent No. 2 treatments declined quite dramatically. All salal leaves were essentially consumed by the end if the fifth day.

*Novel Odors.* To determine if deer were responding to the fecal odors because of their "novel" smell, trials with ammonia and human urine were conducted. These results are shown in Figure 4. Neither ammonia nor human urine suppressed deer feeding as effectively as the jaguar and wolf fecal odors. Ammonia was significantly ($P < 0.01$) more effective than the control on days 2 and 3 but had considerably less (40%) effect than the jaguar feces. Similarly, there was no significant variation between human urine and control in terms of consumption of salal leaves by deer. Both jaguar and wolf feces elicited a highly significant ($P < 0.01$) suppression of feeding compared with the control and urine.

*Predator Fecal Odors—Adhesive Application.* The fifth trial examined an adhesive (Rhoplex acrylic resin) application to maintain the fecal odor around the salal leaves and to provide some degree of protection from rain (Figure 5). All fecal treatments significantly ($P < 0.01$) suppressed deer feeding compared with the control during days 2–5. There was no significant variation among treatments in this trial.

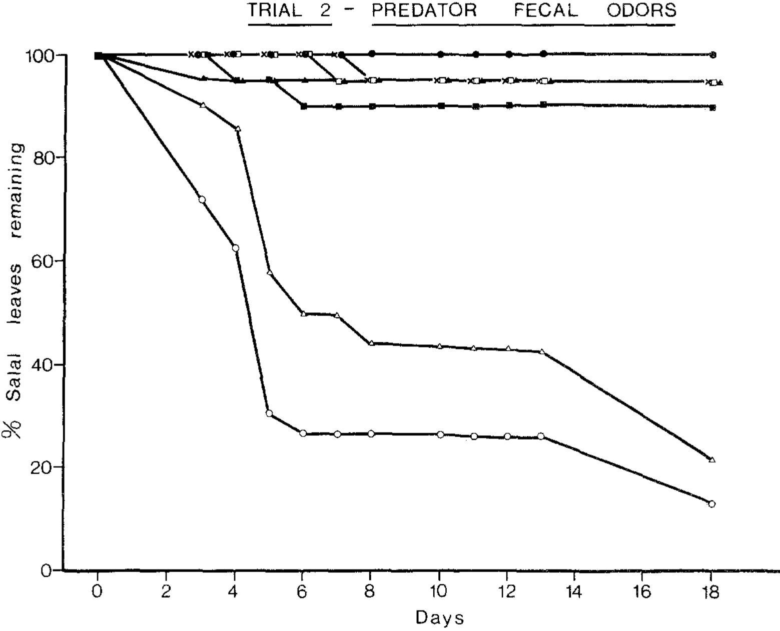


FIG. 2. Effectiveness of predator fecal odors in suppressing deer browsing of salal leaves. Treatments are cougar •, coyote ▲, wolf ■, lynx-bobcat +, commercial repellent No. 1 △, commercial repellent No. 2 □, and control ○.

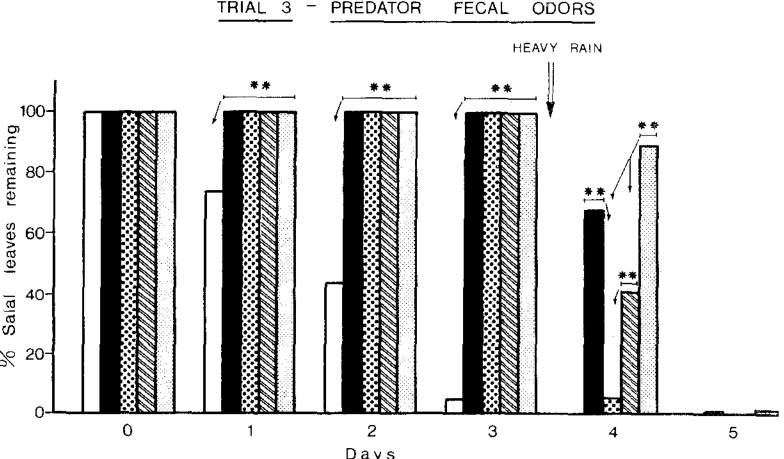


FIG. 3. Effectiveness of predator fecal odors in suppressing deer browsing of salal leaves during adverse weather conditions (** $P < 0.01$, analysis of variance). Treatments are coyote ■, jaguar ⊡, wolf ▧, commercial repellent No. 2 ▨, and control □. Heavy rain fell during a 24-h period between days 3 and 4.

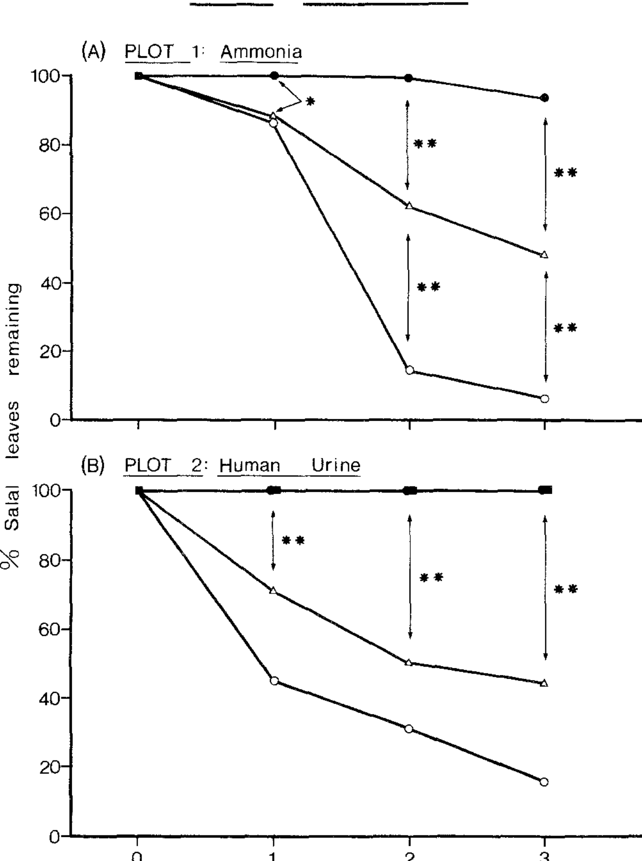


FIG. 4. Effectiveness of novel odors, ammonia and human urine, compared with jaguar and wolf feces in suppressing deer browsing of salal leaves (* $P < 0.05$; ** $P < 0.01$, analysis of variance). Treatments are jaguar ●, wolf ■, ammonia and human urine △, and control ○.

*Predator Fecal Odors—Vial Application.* The continued outstanding effectiveness of fecal odors in association (adhesive application to stake) with salal leaves rather than applied directly on them, suggested that a given predator odor could be dispensed from a vial or capsule system. Figure 6 illustrates a comparison of adhesive and vial applications of fecal odors. Deer consumed significantly ($P < 0.01$) more control salal than any of the treatments for both techniques throughout the trial. In the adhesive application, the efficacy of the wolf fecal odor showed a significant decline on days 4 ($P < 0.05$) and 5 ($P < 0.01$) compared with the coyote and jaguar. In the case of the vial application, the

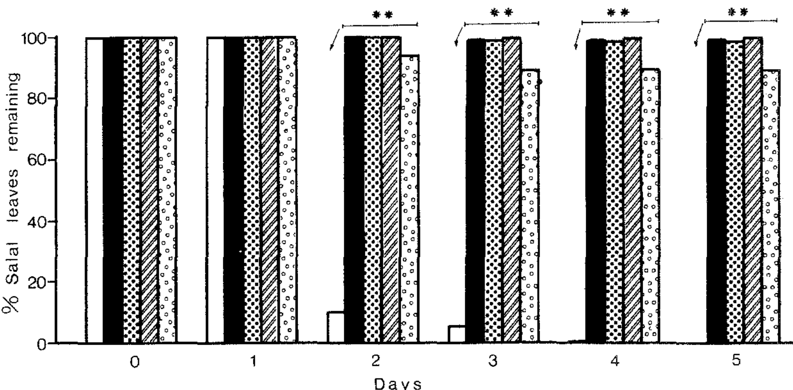


FIG. 5. Effectiveness of predator fecal odors adhesively applied (Rhoplex) to salal leaves in suppressing deer browsing (** $P < 0.01$, analysis of variance). Treatments are coyote ■, jaguar ▦, wolf ▧, cougar ▩, and control □.

coyote and jaguar feces were not as effective as the wolf at day 5 of the trial. However, in general, both techniques suppressed deer feeding on salal to a highly significant degree compared with controls.

*Predator Urine Odor.* The effectiveness of predator urine odor in suppressing deer feeding on salal is illustrated in Figure 7. Control salal leaves were completely consumed after one day. In plot 1, fox and coyote urine were significantly ($P < 0.05$ and $P < 0.01$) more effective than bobcat in reducing deer browsing. However, all the predator urines averaged 84.9% salal remaining compared with 0% control by day 6 in this plot. Similarly, wolf, wolverine, and lynx urines averaged 80.2% salal remaining compared with 0% control at day 6 in plot 2. As expected, deer consumption of treated salal increased dramatically when the vials of urine were removed at the end of day 6. For the cats, urine odor from lynx suppressed deer feeding to a more significant ($P < 0.05$ and $P < 0.01$) degree than that from the bobcat throughout the trial. In general, there was little variation among the other predator urines for within and between plot comparisons.

Of all the predator urines, coyote provided the most consistent suppression of deer browsing and was subsequently tested on conifer seedlings. Figure 8 illustrates the effectiveness of this urine in suppression of feeding on western red cedar and Douglas fir seedlings. After no feeding during the first day of the trial, deer consumed significantly ($P < 0.05$ and $P < 0.01$) more control than treated cedar seedlings. A similar pattern was evident for the Douglas fir, which had significantly ($P < 0.01$) more control than treated seedlings consumed at days 3 and 4. Deer preference for cedar seedlings over those of other conifer

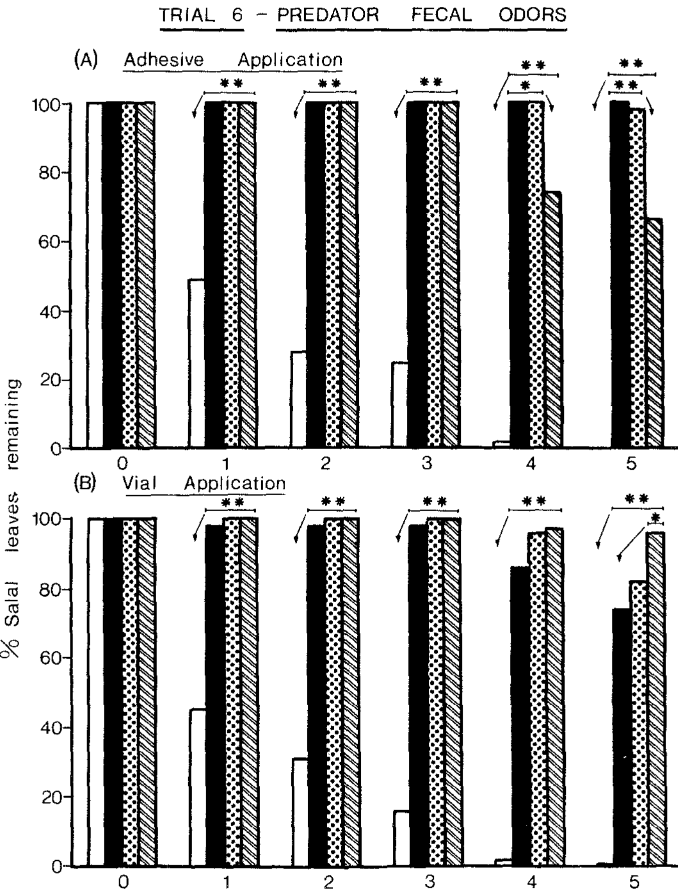


FIG. 6. Suppression of deer browsing on salal leaves with predator fecal odors in adhesive and vial applications (* $P < 0.05$; ** $P < 0.01$, analysis of variance). Treatments are coyote ■, jaguar ⊡, wolf ▧, and control □.

species is clear from this trial where 50% more clear cedar than fir was consumed.

## DISCUSSION

This study was designed to determine the impact of various predator odors on the feeding behavior of deer. As discussed for the snowshoe hare (Sullivan et al., 1984), odors were tested from terrestrial predators (order Carnivora) whose distribution was sympatric with mule deer (*O. hemionus*) in British Columbia. Sources of odor were from feces (Müller-Schwarze, 1972) and urine

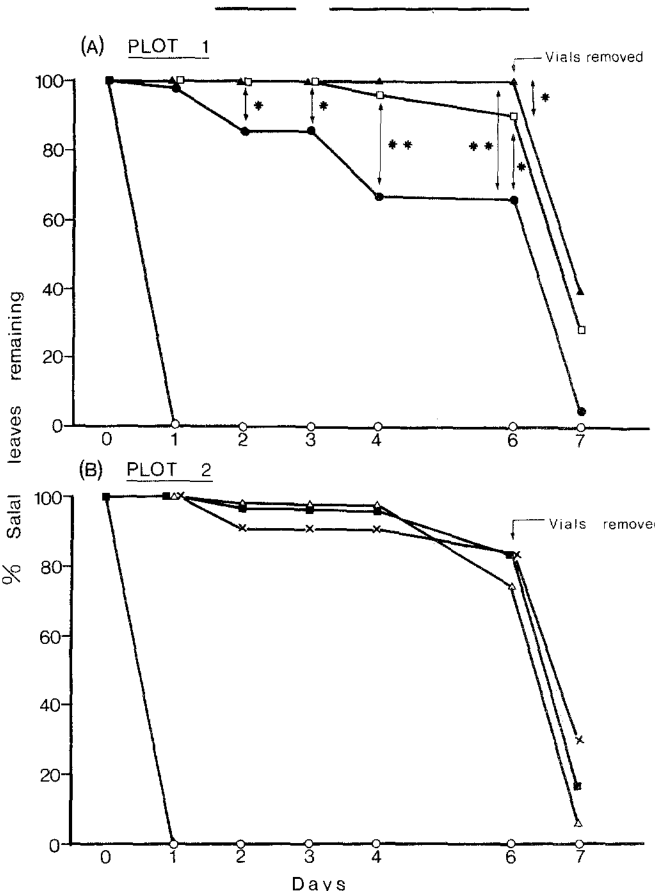


FIG. 7. Effectiveness of predator urine odors (vial application) in suppressing deer browsing of salal leaves (* $P < 0.05$; ** $P < 0.01$, analysis of variance). Treatments are coyote ▲, fox □, bobcat ●, wolf ■, wolverine △, lynx ×, and control ○. Vials were removed at the end of day 6 of the trials.

(Stoddart, 1980a). Results of bioassay testing clearly demonstrated that predator fecal and urine odors suppressed feeding by deer on salal leaves. Similar responses have been recorded in other studies with deer feeding behavior and predator odors (Müller-Schwarze, 1972; Melchiors and Leslie, 1984).

All fecal and urine preparations suppressed deer browsing relative to controls. Cougar, coyote, and wolf are all natural predators of deer, so it is not surprising that their odors were the most effective (see Figures 1, 2, and 7). Lynx–bobcat feces were as effective as other feces but their urines were not

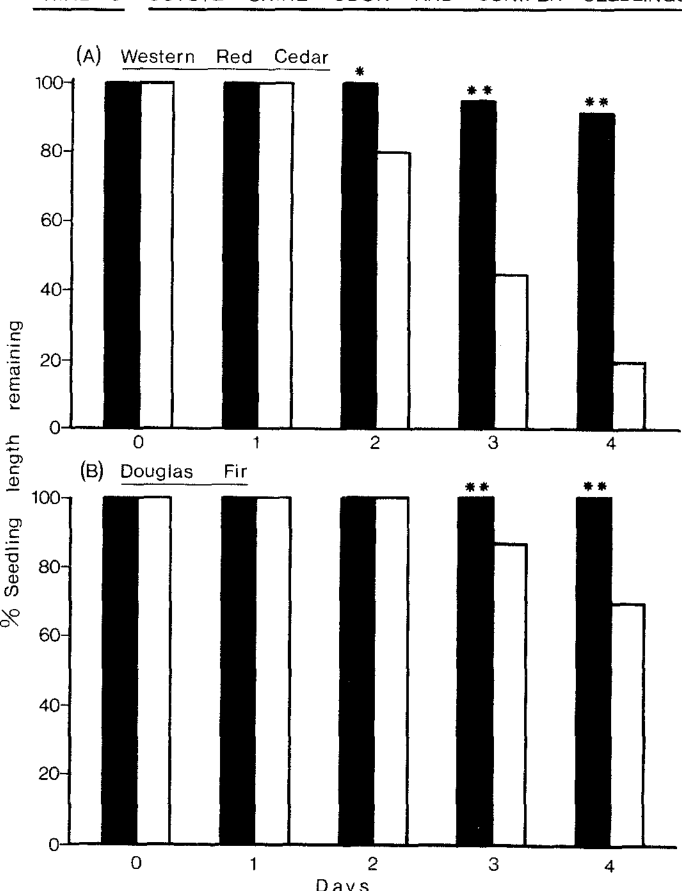


FIG. 8. Suppression of deer browsing on western red cedar and Douglas fir seedlings with coyote urine odor (* $P < 0.05$; ** $P < 0.01$, analysis of variance). Coyote urine treatment represented by shaded and control by unshaded histograms.

(Figure 7). Neither of these cat species normally preys on deer but rather prefer small mammals such as the snowshoe hare (Brand and Keith, 1979). Sullivan et al. (1984) found that cougar, coyote, and wolf feces did not deter hares from feeding. This is in direct contrast to the results with deer and, based on predator–prey specificity, is a predictable response. The urine bioassays with hares were similar to those conducted with deer, where urine of all predator species was effective (Sullivan et al., 1984). In this study, as predicted, the nonpredator lynx and bobcat urines did not reduce deer feeding as much as the other predator species. An exception was the fox urine which suppressed feeding in a similar

manner to the coyote, wolf, and wolverine. The fox, a solitary canid, does not normally prey on deer, but has a particularly potent urine odor (Wilson et al., 1978), which may account for its greater effect relative to lynx and bobcat urines (see Figure 7).

There are two potential criticisms which could affect the interpretation of our results. First, were the responses of deer due to the strangeness or novelty of a given odor? In the presence of ammonia and human urine, deer readily consumed salal at a level comparable to or near control material. Therefore, the suppression of deer feeding was not a response to a novel odor but rather lends support to the hypothesis of a fear response elicited by predator odors. It is interesting to note that fecal odor from the jaguar, a predator species unknown to deer in northwestern North America [although the species are sympatric in southern parts of the continent (Honacki et al., 1982)], was also highly effective in reducing deer browsing. This suggests that deer responded to a signal which indicated "predator" and not just to any novel odor. A comparable study of the effect of weasel and jaguar odors on captive voles reached a similar conclusion (Stoddart, 1980b).

Second, the randomized block design tested all predator odors at a given time. Therefore, there may have been some interaction between odors of different predator species. This interaction may have strengthened the response of deer to the salal leaves, thereby providing the longer term (up to 20 days) results than those recorded for snowshoe hares (see Sullivan et al., 1984). However, from general observations of deer behavior during the trials, a given animal would cautiously approach, sniff the salal, and then start feeding or retreat, depending on the experimental material. Therefore, the distance (6 m) between browse items may have been great enough to minimize mixing and interaction of various odors. This examination of odors at close range by herbivores (deer and hares) suggests that the effective odors may keep the animals from feeding on treated plants but may not exclude them from an entire area (see Müller-Schwarze, 1974). This premise requires further investigation because a large area or block of an effective predator odor may indeed prevent deer or hares from entering that area, particularly if the habitat provides poor cover (e.g., young forestry plantation or tree fruit orchard).

In the study with snowshoe hares and predator odors, Sullivan et al. (1984) formulated the supposition that the short-term (up to seven days) responses were due to evaporative loss of the active components of a given predator sample. If this was the case, why did deer not respond in a similar manner? Deer may have a lower threshold fear response to the odor of a predator because of their lack of numbers (about 1–5/km$^2$) (Harestad and Jones, 1981) compared with a peak population of snowshoe hares (up to 850/km$^2$) (Keith and Windberg, 1978). Compared with hares, these ungulates may respond to lower concentrations of the active components or possibly to different compounds. Deer readily con-

sumed salal when predator extracts were washed away by rain (see Figure 3) or when the vials were removed as in trial 7 (see Figure 7). This clearly indicates that some aspect of the predator odors was in effect throughout trials 1 and 2.

Another related explanation is that the 0.6-hectare enclosure may have allowed deer more room to "escape" from the odors compared with the smaller hare enclosure (see Sullivan et al., 1984). An investigation of the response of a low population of hares to predator scent would be an interesting comparison with the deer study. There may be variation in the fear response threshold of hares (and perhaps deer in varying habitats and years) during different phases of the 10-year cycle. Such variation might arise from the potential refinement that learning processes impose on the largely genetically determined negative response to predator odors (Lockley, 1964; Müller-Schwarze, 1972; Henessy and Owings, 1978).

Bioassay trials 1 and 2 provided long-term results because the negligible rain fall during these periods did not dilute the treatments. The cougar, coyote, wolf, and lynx–bobcat feces suppressed feeding by deer on a level comparable with commercial repellent No. 2, which was specifically developed as a big game repellent. However, this repellent is subject to dilution from rain as illustrated in Figure 3 where all treated salal was essentially consumed after five days. The advantages of predator odors as repellents lie in their biological ability to produce a fear response and their potential encapsulation in controlled-release devices. This study has shown that predator odors are equally effective in direct foliar application, adhesive application, and in plastic vials, which are essentially crude controlled-release capsules. Therefore, as discussed for snowshow hares (Sullivan et al., 1984), the active repellent components of predator odors which suppress deer feeding may also be encapsulated for optimal control of evaporation rate over time. This process could provide long-term efficacy and durability as well as flexibility in application of a given repellent to forestry plantations and agricultural crops.

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# JUGLONE CONCENTRATION IN SOIL BENEATH BLACK WALNUT INTERPLANTED WITH NITROGEN-FIXING SPECIES

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**Abstract**—Juglone concentration was measured in soils collected under a 14-year-old black walnut (*Juglans nigra* L.) plantation containing plots of pure walnut and of walnut mixed with either autumn-olive (*Elaeagnus umbellata* Thunb.) or European black alder (*Alnus glutinosa* (L.) Gaertn.). Juglone concentrations declined with soil depth and distance from the walnut tree. Concentrations in autumn-olive–walnut plots were statistically lower than concentrations in European black alder–walnut or pure walnut plots. The concentration of juglone in soil in European black alder–walnut plots was apparently sufficient to cause the onset of black alder mortality.

**Key Words**—Allelopathy, black walnut, *Juglans nigra* L., thin-layer chromatography, mixed plantings.

## INTRODUCTION

Black walnut (*Juglans nigra* L.) produces a phytotoxic phenolic compound called juglone (5-hydroxy-1,4-naphthoquinone). In addition to causing plant mortality, juglone may affect the ecology of a plant community by causing changes in species composition, rates of plant succession, and growth rates (Rietveld, 1981). Not all plants are affected by juglone (Brooks, 1951). Even though there are numerous reports in the literature on the effects of black walnut on other plants (MacDaniels and Pinnow, 1976; Rietveld, 1983; Rietveld et al., 1983), no information exists on the relation between juglone concentration and effects on plant growth in the field.

Research has shown that mixed plantings of certain nitrogen-fixing plants

with black walnut in plantations can substantially increase walnut growth and possibly quality (Funk et al., 1979; Schlesinger and Williams, 1984). However, little is known about the long-term effects of juglone on the interplanted species. Rietveld (1981) presented evidence suggesting that, when walnut and other species are established together in plantations, juglone concentrations build up for 12–25 years before allelopathic effects become evident, if they occur at all. Later, Rietveld et al. (1983) hypothesized that the juglone concentration in the soil is related to the rate of juglone production and release by black walnut trees and the rate of breakdown by aerobic heterotrophs. The better the soil aeration, the higher the level of aerobic metabolism by soil microorganisms and the lower the juglone concentration.

The purpose of this study was to determine the concentration and gradient of juglone in soil beneath plantation black walnut planted in mixture with nitrogen-fixing nurse species.

## METHODS AND MATERIALS

The study was conducted on an upland site in Hardin County, southern Illinois, on the Kaskaskia Experimental Forest of the U.S. Forest Service. Soils on the broad upland site (7–10% slopes) are of the Hosmer series (Typic Fragiydalfs; fine silty, mixed, mesic). The soils are moderately well drained and are 2–3 m thick over sandstone bedrock.

In the spring of 1969, nursery grown autumn-olive (*Elaeagnus umbellata* Thunb.) and European black alder (*Alnus glutinosa* (L.) Gaertn.) were interplanted alternately with 1-0 black walnut stock in 29.6- × 38.4-m plots. Rows are 3.7 m apart. Within rows of pure walnut plots, walnut is spaced 4.9 m apart; in interplanted plots, the walnut trees and autumn-olive or European alder trees are spaced 2.4 m apart, thus achieving a 1 : 1 mixture of walnut to interplanted species. By the fall of 1982, height of black walnut trees ranged from 1.5 m in walnut-only plots to 5.5 m in mixed plots. Nitrogen-fixing plants averaged 5 and 7 m for autumn-olive and European black alder, respectively.

Soil samples were collected in mid-November of 1982 near five black walnut trees in each treatment plot of walnut-only, European alder and walnut, and autumn-olive and walnut. A total of 45 trees (three replications) were used. Surface litter was carefully removed to mineral soil before removing soil samples with a soil sampling tube at distances of 0.9 and 1.8 m from each black walnut tree in the five-tree row plot. Depth of sampling included 0–8, 8–16, 16–30, and 52–61 cm at 0.9 m and 0–8 cm and 8–16 cm at 1.8 cm. Samples were taken from within rows of walnut trees, immediately transported to the laboratory, and stored at 4°C until analyzed.

In the laboratory, soils were sieved through a 40-mesh sieve and dried at 40°C for 24 hr. Duplicate samples containing 50 g of sieved soil from each

sample were placed in a 250-ml Erlenmeyer flask containing 100 ml of chloroform, and shaken at 200 oscillations per minute for 30 min. The mixture was filtered through a glass crucible filter. The filtered soil sample was shaken with two successive 70-ml volumes of chloroform and filtered after each. The filtrates from the three successive extractions were combined and concentrated to 2 ml in a rotary evaporator at 50°C. A 0.25-ml aliquot was banded on a silica gel G TLC plate and chromatographed with a solvent mixture of cyclohexane, chloroform, and glacial acetic acid (70 : 20 : 10). Juglone was observed as a visible yellow-orange band at $R_f = 0.40$ (Hedin et al., 1979). The juglone band was scraped into a test tube, eluted from the silica gel with 8 ml of chloroform, and filtered. The absorbance of the yellow-orange solution was measured with a spectrophotometer at 420 nm.

Juglone concentrations were determined using a standard curve, which was based on a standard solution prepared by dissolving 10 mg of juglone (Aldrich Chemical Co., Milwaukee, Wisconsin) in 250 ml of chloroform in a volumetric flask. The standard solution contained 40 $\mu$g of juglone per milliliter of chloroform. A series of volumes, 0.05, 0.1, 0.15, 0.2, and 0.3 ml each of the standard solution were banded on a silica gel G TLC plate and chromatographed with the solvent mixture as described above. The absorbance values for the soil–chloroform extracts were compared to the standard curve and the amount of juglone calculated.

Analyses of variance models were used to determine differences associated with treatments, distance from tree, soil depth, and their interactions.

## RESULTS AND DISCUSSION

The mean juglone concentration in soil beneath plots of autumn-olive–black walnut differed significantly from the concentration of juglone in soils beneath European black alder–black walnut and black walnut-only plots (Table 1). Juglone concentrations also differed with sampled depth and distance from the walnut tree, but only the 0- to 8- and 52- to 61-cm depths differed significantly at 0.9 m from the tree. Higher concentrations were present at the 0- to 8-cm depth and 0.9 m from the tree than at lower depths or at the same depth 1.8 m from the walnut tree. This is due to the proximity of leaves, fruit, and roots within a few centimeters of the soil surface and within the 0.9 m area; all plant parts contain initially high juglone levels (MacDaniels and Pinnow, 1976).

Higher amounts of juglone were detected in the soil at the 8- to 16- and 16- to 30-cm depths in the walnut-only treatment in comparison with mixed treatments; juglone levels between mixed treatments at these soil depths were similar (Table 2). Soil environmental conditions in mixed plots are apparently more conducive to the breakdown of juglone than are conditions in walnut-only plots. Both Fisher (1978) and Rietveld (1981) suggested that soil moisture affects

TABLE 1. MEAN JUGLONE CONCENTRATION IN SOIL COLLECTED FROM BLACK WALNUT TREES IN A 14-YEAR-OLD MIXED PLANTING

| Treatment | | Mean Juglone Conc. ($\mu$g/g Soil) |
|---|---|---|
| Black alder and walnut | | 1.23 a[a] |
| Autumn-olive and walnut | | 0.36 b |
| Walnut-alone | | 1.76 a |
| **Distance (m)** | **Depth (cm)** | |
| 0.9 | 0–8 | 2.78 a |
| | 8–16 | 0.88 ab |
| | 16–30 | 0.78 ab |
| | 52–61 | 0.02 b |
| 1.8 | 0–8 | 1.36 a |
| | 8–16 | 0.27 b |

[a]Means followed by the same letter are not significantly different at the 0.05 level according to Scheffe's method of contrast.

TABLE 2. JUGLONE CONCENTRATION IN SOIL ACCORDING TO DEPTH, DISTANCE, AND TREATMENT IN A 14-YEAR-OLD MIXED PLANTING

| | | Juglone Conc. ($\mu$g/g Soil) | | |
|---|---|---|---|---|
| Distance (m) | Depth (cm) | Black Alder and Walnut | Autumn-olive and Walnut | Walnut Only |
| 0.9 | 0–8 | 3.95 a[a] | 1.85 a | 3.65 a |
| | 8–16 | 0.55 b | 0.50 ab | 1.80 ab |
| | 16–30 | 0.35 b | 0.20 ab | 1.60 ab |
| | 52–61 | 0.05 b | 0.00 b | 0.00 b |
| 1.8 | 0–8 | 0.75 a | 0.70 a | 1.55 a |
| | 8–16 | 0.00 b | 0.15 b | 0.50 b |

[a]Means followed by the same letter are not significantly different from the 0.05 level according to Scheffe's method of contrast.

the degradation of juglone and in turn the allelopathic effects of walnut on other species. When planted with black walnut, pines (*Pinus* spp.) were suppressed or died on imperfectly drained soil (Fisher, 1978). In the laboratory, he found that juglone and its inhibitory activity readily disappeared from soil under a dry moisture regime, but was unaffected in the same soil under a wet moisture regime.

The lower mean juglone concentration in soil under autumn-olive compared with European alder (Table 1) is probably associated with edaphic and microenvironmental conditions that enhance microorganisms responsible for the breakdown of plant parts and consequently lead to the breakdown of juglone in the soil. The European black alder was taller than the autumn-olive and often taller than the walnut. Since autumn-olive is a shrub species, it was usually shortest of all three species. The canopy in plots interplanted with European black alder was more open than in plots mixed with autumn-olive. More herbaceous vegetation was present in open-crowned European black alder plots than in autumn-olive plots. Ground cover in autumn-olive plots consisted of small herbaceous plants and a 2- to 3-cm-thick leaf litter layer. There were many clumps of fescue (*Festuca arundinacea* Schreb.) and other grasses present in European black alder plots. The shade and closed canopy provided by autumn-olive reduces water loss due to transpiration and evaporation immediately above the soil, keeping temperatures about 2°C lower in the summer and about 3°C warmer in the winter (Funk et al., 1979). All these conditions were believed to be responsible for the larger number and diversity of microorganisms in soil beneath autumn-olive plots as compared with mixed European black alder plots and walnut-only plots (Mariann Kienzler, personal communication).

These results demonstrate that a juglone concentration gradient exists both downward in the soil and away from the walnut tree. The higher levels of juglone in the black alder and walnut plots may explain the onset of European black alder mortality in the spring prior to our sampling. The amount of juglone in the soil apparently has reached a toxic level for this species. The growth of hydroponically grown European black alder and autumn-olive was depressed when grown in solutions containing juglone at a strength of $10^{-5}$ M (Rietveld, 1981). By comparison, our data show twice this amount [0.0017416 g juglone/liter (Rietveld, 1981) compared to 0.00365 g and 0.00395 g juglone/1000 g soil, respectively]. However, the actual concentrations of juglone that the European black alder and autumn-olive are exposed to in the soil solution are probably less than the amounts found in our analysis because of juglone's high solubility in chloroform. We conclude that autumn-olive will persist in the mixture with black walnut for some time before the effects of juglone toxicity become evident.

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# MONOTERPENE OVIPOSITION STIMULANTS OF *Dioryctria amatella*[1] IN VOLATILES FROM FUSIFORM RUST GALLS AND SECOND-YEAR LOBLOLLY PINE CONES

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**Abstract**—Volatiles collected on Poropak Q from fusiform rust galls (*Cronartium quercuum* F. sp. *fusiforme*) of loblolly pine for 3 hr were better oviposition stimulants for *Dioryctria amatella* (Hulst) females than extracts of 8-hr collections. GLC analysis of these extracts showed no major differences in relative monoterpene composition, although 8-hr collections contained an unidentified compound not detected in the 3-hr collections. Comparison of volatiles from second-year loblolly pine cones with those from fusiform galls showed that both contained *dl*-$\alpha$-pinene, $(-)$-$\beta$-pinene, myrcene, and $(+)$-limonene. Camphene was found in galls only, and relatively large quantities of $\beta$-phellandrene were identified only from cones. The five major monoterpenes found in the two host substrates were tested in an oviposition bioassay. Eighteen trials using different combinations of these terpenes showed that the combination of $\alpha$-pinene, myrcene, and limonene was as attractive to *D. amatella* females as all other terpene combinations, including turpentine.

**Key Words**—*Dioryctria amatella*, Lepidoptera, Pyralidae, oviposition stimulants, monoterpenes, $\alpha$-pinene, myrcene, limonene.

## INTRODUCTION

Monoterpenes, which are major constituents of the oleoresin in coniferous trees, are used by a variety of insects for host finding and recognition (Heikkenen and

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Hrutfiord, 1965; Rudinsky, 1966; Thomas and Hertel, 1969; Werner, 1971; Alfaro et al., 1980). Kossuth and Munson (1981) listed 15 monoterpenes regularly found in southern pines, but only five are abundant in loblolly pine (*Pinus taeda* L.) (Squillace and Wells, 1981), an important host of the southern pine coneworm, *Dioryctria amatella* (Hulst). The host substrates of *D. amatella* include fusiform rust galls (*Cronartium quercuum* (Berk.) Miy. ex Shirai F. sp. *fusiforme*), tree wounds, and second-year cones (Coulson and Franklin, 1970). Fatzinger (1981) noted that *D. amatella* females would not oviposit unless host substrate was present. These studies suggest that olfactory cues may be important in locating oviposition sites. The identification of these host attractants would be useful in further studies of *D. amatella* biology. Therefore, we designed a study to determine if volatiles emitted from two loblolly pine host structures stimulate *D. amatella* oviposition. We also sought to identify and test suspected oviposition stimulants in a laboratory bioassay.

## METHODS AND MATERIALS

*Volatile Collection, Testing, and Identification.* Volatiles from fusiform rust galls and second-year cones were collected on Porapak Q (Peacock et al., 1975). Rust galls cut from the main stems of six loblolly pine trees (1–3 m height) in late March 1981 were placed in a metal container (Figure 1) 1 hr after cutting without treating the cut surface. Air was drawn through the container and through a conditioned Porapak Q column (Byrne et al., 1975) for 3 hr at a flow rate of ca. 8 liters/min. This first column was replaced after 3 hr with a second column on which volatile collection continued for an additional 8 hr.

Cone volatiles were similarly collected in June 1982. Four loblolly pine clones were selected from a pine seed orchard. A total of 10 cones per clone were removed from several ramets and placed in glass desiccator chambers from which volatiles were collected for 3 hr. Volatiles were collected separately from each clone.

Volatiles were recovered from the Porapak Q by Soxhlet extraction with 200 ml pentane for 24 hr (Byrne et al., 1975). The extracted material was concentrated to ca. 2 ml in a rotary evaporator.

Extracts of the fusiform canker volatiles were tested as possible oviposition stimulants in 12 wire screen cages (30 × 34 × 38 cm). Each cage contained two dispensers of 5% dextrose solution plus 10 male and 10 female *D. amatella* moths that had been reared on an artificial diet (Fedde, 1982). Clear polyethylene was stapled to the sides and bottoms of each cage to prevent movement of volatiles to adjacent cages. The cages were kept in a windowless room maintained on a 16-hr–8-hr light–dark cycle. The temperature was maintained at 24–27°C and the relative humidity at ca. 50%.

Extracts from 3-hr and 8-hr Porapak collections were assayed by placing 100 $\mu$l of concentrate in a slow-release dispenser similar to that described by

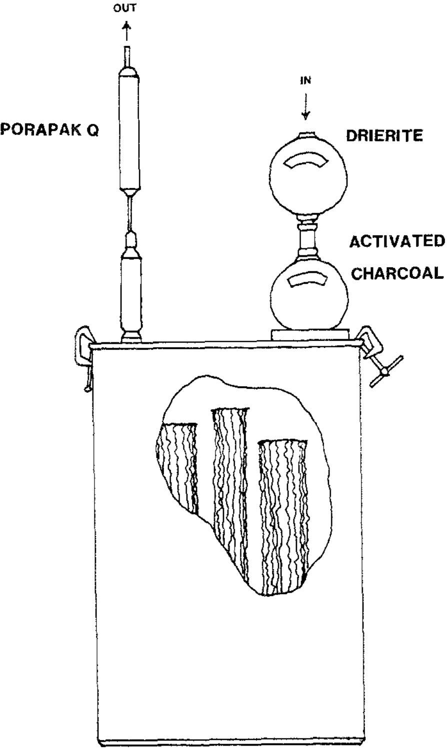


FIG. 1. Diagram of the apparatus used to collect volatiles from freshly cut fusiform rust galls.

Billings et al. (1981). Each dispenser consisted of a 2-dram vial with a plastic stopper in which a 3-mm hole had been drilled. The concentrate was placed on a cotton roll (1 × 2 cm) located inside the vial, which was then covered with a fine mesh cloth sleeve. The ends of the sleeve were closed with plastic-coated wire ties that were bent to form "legs" which held the cloth-covered vial ca. 1 cm above the cage floor.

Three dispensers were randomly assigned to the front, middle, or back of each cage. Each dispenser contained an extract of either the 3- or 8-hr Porapak Q collections, or a 1-ml pentane check. The experiment included two trials, one with 12 and one with four replicates. Each trial lasted for two weeks; then any eggs deposited on the dispensers were removed and counted.

Volatiles collected on Porapak Q were identified by gas chromatography on a Tracor 222 gas chromatograph connected to a Hewlett Packard integrator (model 3390A). Samples and terpene standards were injected on a 3.7-m glass column packed with 30% Carbowax 20 M on Chromosorb W (60–80 mesh) at an oven temperature of 95°C and a $N_2$ carrier gas flow rate of 25 ml/min.

*Monoterpene Oviposition Bioassay.* Standards of the five major monoter-

penes of loblolly pine were tested for ability to induce oviposition in the same manner as Porapak Q extracts, except that the three dispensers were placed equidistantly across the front of the cages to reduce variation due to position, and the pentane checks were always positioned in the center between two treatment vials. Also, the cages and humidifier were placed under a clear polyethylene cover to increase the humidity in the cages to 70%.

Eighteen trials of various terpene combinations were run from August 1982 to June 1983. Initially, we compared three quantities (50, 100, and 150 $\mu$l) of *dl*-$\alpha$-pinene without a pentane check. Later we compared different quantities (15, 75, or 150 $\mu$l) and combinations of (−)-$\beta$-pinene, myrcene, (+)-limonene, and $\beta$-phellandrene. A pure sample of $\beta$-phellandrene could not be obtained so Unitene (Union Camp Corp., Terpene and Aromatics Division, P.O. Box 37617, Jacksonville, Florida 32236), a 1:1 mixture of limonene and $\beta$-phellandrene (95% pure), was compared to vials containing $\alpha$-pinene and limonene. Finally, combinations of $\alpha$-pinene, myrcene, limonene, and $\beta$-phellandrene were compared to 300 $\mu$l of raw turpentine distilled from oleoresin (Filtered Resin Products, Baxley, Georgia 31513).

Terpenes in all trials were formulated in a final volume of 1 ml of pentane–terpene mixture. The turpentine was analyzed by gas chromatography to determine its relative content of $\alpha$-pinene; then enough turpentine (in pentane) was added to the dispensers so that each contained ca. 150 $\mu$l of $\alpha$-pinene plus the remaining terpenes.

Treatment means were analyzed by ANOVA and ranked by Duncan's new multiple-range test.

## RESULTS

Bioassays of volatiles from fusiform rust cankers showed that dispensers containing extracts of the 3-hr Porapak Q collections had significantly more eggs ($P < 0.05$) than those containing the 8-hr collections or pentane checks (Table 1). Chemical analysis (Table 2) showed that 3-hr and 8-hr collections were almost identical in relative monoterpene composition. It is unclear why the 8-hr collection was less attractive. Byrne (1975) reported that certain highly volatile compounds "break through" Porapak columns when collections are made over extended periods. However, breakthrough appears unlikely since the 3-hr and the 8-hr collections had similar monoterpene composition. Several unknown compounds that accumulated in the 8-hr collection may have inhibited oviposition, but more work is needed to determine their identity and importance.

Monoterpene volatiles from second-year loblolly pine cones differed from cankers in the relative proportions of $\alpha$-pinene, $\beta$-pinene, and myrcene (Table 2). In addition, cankers produced small quantities of camphene but no $\beta$-phellandrene, while cone volatiles contained no detectable camphene but contained

TABLE 1. MEAN NUMBER OF *D. amatella* EGGS DEPOSITED ON OVIPOSITION VIALS CONTAINING 100 $\mu$l OF CONCENTRATED EXTRACTS OF PORAPAK Q COLLECTIONS FROM FUSIFORM RUST CANKERS[a]

| Duration of porapak Q collection (hr) | Quantity of pentane extract ($\mu$l) | Mean no. of *D. amatella* eggs[b] Trial 1 ($N = 12$) | Trial 2 ($N = 4$) |
|---|---|---|---|
| 0 | 100 | 0.5[a] | 2.0[a] |
| 3 | 100 | 5.3[b] | 140.5[b] |
| 8 | 100 | 0.7[a] | 37.8[a] |

[a]Canker volatiles were collected over periods of 3 and 8 hr.
[b]Within each trial, means followed by the same letter are not significantly different by Duncan's new multiple-range test ($P < 0.05$).

ca. 9% $\beta$-phellandrene. The lack of $\beta$-phellandrene in fusiform rust canker volatiles is consistent with the findings of Squillace and Wells (1981), who showed a tendency for trees with low $\beta$-phellandrene content to be more susceptible to fusiform rust infection.

Mean numbers of *D. amatella* eggs laid on dispensers were influenced by the monoterpene present in the containers (Table 3). No significant differences ($P < 0.05$) were found in the numbers of eggs deposited on dispensers con-

TABLE 2. MONOTERPENE CONTENT OF VOLATILES COLLECTED ON PORAPAK Q FROM FUSIFORM RUST CANKERS AND SECOND-YEAR LOBLOLLY PINE CONES, TWO HOST SUBSTRATES OF *Dioryctria amatella*, DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

| | Porapak Q collections (% content) | | |
|---|---|---|---|
| | Fusiform rust cankers | | Loblolly pine cones[a] |
| Volatile compounds | 3-hr Collection | 8-hr Collection | 3-hr Collection |
| $\alpha$-Pinene | 86.6 | 83.9 | 66.2 |
| Camphene | 0.6 | 0.8 | |
| $\beta$-Pinene | 11.1 | 13.1 | 21.1 |
| Myrcene | 0.7 | 0.9 | 2.6 |
| Limonene | 0.9 | 1.2 | 1.1 |
| $\beta$-Phellandrene | | | 8.9 |
| Unknown | | 0.2 | |

[a]Means of four different loblolly pine clones.

TABLE 3. MEAN NUMBERS OF EGGS LAID BY 10 *Dioryctria amatella* FEMALES ON SLOW-RELEASE DISPENSERS CONTAINING VARIOUS COMBINATION OF 5 MONOTERPENES FOUND IN LOBLOLLY PINES

| Trial | Content of monoterpene dispensers[a] | | | | Mean no. of *D. amatella* eggs[b] | | |
|---|---|---|---|---|---|---|---|
| no. | A | B | C | Replications | A | B | C |
| 1 | 150 μl α-pinene | 100 μl α-pinene | 50 μl α-pinene | 12 | 136.7[a] | 89.7[a] | 85.7[a] |
| 2 | 150 μl α-pinene | 150 μl β-pinene | 1 ml pentane | 9 | 82.6[a] | 111.6[a] | 20.1[b] |
| 3 | 150 μl α-pinene | 150 μl α-pinene + 75 μl β-pinene | 1 ml pentane | 12 | 202.8[a] | 237.8[a] | 78.3[b] |
| 4 | 150 μl α-pinene | 150 μl myrcene | 1 ml pentane | 6 | 66.3[a] | 316.7[b] | 79.0[a] |
| 5 | 150 μl α-pinene | 150 μl α-pinene + 15 μl myrcene | 1 ml pentane | 6 | 90.7[a] | 347.8[b] | 79.3[a] |
| 6 | 150 μl α-pinene | 150 μl α-pinene + 75 μl myrcene | 1 ml pentane | 6 | 69.2[a] | 373.3[b] | 71.0[a] |
| 7 | 150 μl α-pinene | 150 μl limonene | 1 ml pentane | 6 | 180.0[a] | 151.7[a] | 93.7[a] |
| 8 | 150 μl α-pinene | 150 μl α-pinene + 15 μl limonene | 1 ml pentane | 6 | 73.3[a] | 315.5[b] | 42.5[a] |
| 9 | 150 μl α-pinene | 150 μl α-pinene + 75 μl limonene | 1 ml pentane | 6 | 82.8[a] | 309.3[b] | 69.0[a] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | 150 μl α-pinene + 75 μl myrcene | 150 μl α-pinene + 75 μl myrcene + 75 μl limonene | 1 ml pentane | 4 | 185.5[a] | 375.0[b] | 33.7[c] |
| 11 | 150 μl α-pinene + 75 μl limonene | 75 μl limonene + 75 μl β-phellandrene | 1 ml pentane | 5 | 244.0[a] | 236.2[a] | 49.0[b] |
| 12 | 150 μl α-pinene + 15 μl limonene | 150 μl α-pinene + 15 μl limonene + 15 μl β-phellandrene | 1 ml pentane | 6 | 122.0[a] | 232.5[b] | 35.0[a] |
| 13 | 150 μl α-pinene + 75 μl limonene | 150 μl α-pinene + 75 μl limonene + 75 μl β-phellandrene | 1 ml pentane | 6 | 114.5[a] | 227.0[b] | 48.2[c] |
| 14 | 150 μl α-pinene + 75 μl myrcene + 75 μl limonene | 150 μl α-pinene + 75 μl myrcene + 75 μl limonene + 75 μl β-phellandrene | 1 ml pentane | 6 | 262.0[a] | 256.6[a] | 60.4[b] |

[a]Dispensers contained the stated quantities of monoterpenes in 1 ml pentane. Vials containing β-phellandrene were prepared by adding Unitene (Union Camp Corp.), a 1:1 mixture of limonene and β-phellandrene.
[b]Within each trial, means followed by the same letter are not significantly different ($P < 0.05$) by Duncan's new multiple-range test.

taining 50, 100, or 150·μl of α-pinene (trial 1). However, since *D. amatella* females tended to oviposit more often on dispensers containing 150 μl of α-pinene, that quantity was used as a standard for comparison in the remaining trials. No oviposition preference was evident for dispensers containing β-pinene alone (trial 2) or in combination with α-pinene (trial 3) over α-pinene alone, suggesting that female *D. amatella* may not be able to differentiate between the two isomers. However, females laid more eggs on dispensers containing a combination of α-pinene and myrcene than on β-pinene and myrcene (Table 4). Females exhibited a similar preference for α-pinene and limonene over dispensers containing a combination of β-pinene and limonene.

Myrcene alone (Table 3, trial 4) or in combination with α-pinene, regardless of the α-pinene–myrcene ratio (trials 5 and 6), significantly increased oviposition over that with α-pinene alone. In contrast, egg deposition did not vary significantly between dispensers of limonene alone (trial 7) and α-pinene alone. However, females oviposited ca. three to four times more often on dispensers containing combinations of α-pinene and limonene than they did on dispensers of α-pinene alone (trials 8 and 9). Limonene combined with α-pinene and myrcene (trial 10) was preferred over the combination of α-pinene and myrcene. Dispensers with β-phellandrene plus limonene had as many eggs as those with α-pinene plus limonene (trial 11). However, dispensers with limonene and β-phellandrene in combination with α-pinene (trials 12 and 13) had significantly more eggs than those with only α-pinene and limonene. The addition of β-phellandrene did not enhance oviposition over that observed for the combination of α-pinene, limonene, and myrcene (trial 14).

When turpentine was compared to combinations of the individual monoterpenes found in loblolly pine cones (Table 5), females oviposited more fre-

TABLE 4. MEAN NUMBER OF EGGS LAID BY 10 *Dioryctria amatella* FEMALES ON SLOW-RELEASE DISPENSERS CONTAINING VARIOUS COMBINATIONS OF α-PINENE, β-PINENE, MYRCENE, AND LIMONENE

| Trial no. | Content of monoterpene dispensers[a] | | | | Mean no. of *D. amatella* eggs[b] | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | *N* | A | B | C |
| 1 | 150 μl α-pinene + 75 μl myrcene | 150 μl β-pinene + 75 μl myrcene | 1 ml pentane | 5 | 214.0[a] | 137.8[b] | 33.8[c] |
| 2 | 150 μl α-pinene+ 75 μl limonene | 150 μl β-pinene + 75 μl limonene | 1 ml pentane | 6 | 85.5[a] | 56.3[b] | 8.5[c] |

[a]Dispensers contained the stated quantities of monoterpenes in 1 ml pentane.
[b]Within each trial, means followed by the same letter are not significantly different ($P < 0.05$) by Duncan's new multiple-range test.

TABLE 5. COMPARISONS OF MEAN NUMBERS OF EGGS LAID ON SLOW-RELEASE DISPENSERS CONTAINING TURPENTINE TO VARIOUS COMBINATIONS OF INDIVIDUAL MONOTERPENES

| Trial no. | Content of oviposition vials[a] | | | | Mean no. of *D. amatella* eggs[b] | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | *N* | A | B | C |
| 1 | 300 μl turpentine | 150 μl α-pinene + 75 μl limonene + 75 μl β-phellandrene | 1 ml pentane | 6 | 271.5[a] | 138.7[b] | 84.7[b] |
| 2 | 300 μl turpentine | 150 μl α-pinene + 75 μl myrcene | 1 ml pentane | 5 | 364.2[a] | 209.3[a] | 111.2[b] |
| 3 | 300 μl turpentine | 150 μl α-pinene + 75 μl myrcene + 75 μl limonene | 1 ml pentane | 5 | 295.0[a] | 236.2[a] | 16.4[b] |
| 4 | 300 μl turpentine | 150 μl α-pinene + 75 μl myrcene + 75 μl limonene + 75 μl β-phellandrene | 1 ml pentane | 6 | 338.7[a] | 310.1[a] | 103.8[b] |

[a]Vials contained the stated terpene quantities in 1 ml pentane. Vials containing β-phellandrene were prepared by adding Unitene (Union Camp Corp.), a 1:1 mixture of limonene and β-phellandrene which was 94% pure.
[b]Within each trial, means followed by the same letter are not significantly different ($P < 0.05$) by Duncan's new multiple-range test.

quently on dispensers containing turpentine than on those with α-pinene, limonene, and β-phellandrene (trial 1) or α-pinene and myrcene (trial 2). There was, however, no significant difference between the dispensers with turpentine and those of the combination of limonene, α-pinene, and myrcene (trial 3). Dispensers with α-pinene, myrcene, limonene, and β-phellandrene also had as many eggs as those with turpentine (trial 4).

## DISCUSSION

Of the five monoterpenes found in loblolly pine cone volatiles, α-pinene, myrcene, and limonene appear to be most important as oviposition stimulants of *D. amatella*. Although α-pinene appears to be important in the recognition of oviposition substrates, egg laying is greatly enhanced by the addition of other monoterpenes, especially myrcene and limonene. Females prefer dispensers containing myrcene over those without it.

These oviposition stimulants may be useful in further studies of *D. ama-*

*tella* biology. Since *D. amatella* eggs have never been found within the host trees, an artificial oviposition substrate would be very useful in determining periods of peak oviposition activity. Also, in limited trials monoterpenes served as long-range attractants for both male and female moths (Hanula, unpublished data), suggesting that they could be useful in trapping females to determine periods of peak female activity without the use of light traps. However, more work is needed to determine the potential utility of these chemicals.

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# CAPILLARIES AS CONTROLLED RELEASE DEVICES FOR INSECT PHEROMONES AND OTHER VOLATILE SUBSTANCES—A REEVALUATION

## Part I. Kinetics and Development of Predictive Model for Glass Capillaries[1]

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(Recieved June 25, 1984; accepted October 24, 1984)

**Abstract**—Controlled release formulations are required for the dissemination of behavior-modifying chemicals in insect control strategies. Among the types of formulations that have been used for some time are glass and plastic capillaries. Erratic release rates on field use of such capillaries prompted us to reexamine the release of volatile materials with regard to (1) kinetics of the release, (2) the effect of the vapor–air column above the liquid, and (3) developing a predictive model. Results indicate that the release is not zero order, and that the length of the vapor–air column is a critical factor of the system; a predictive model has been developed that will allow better design of capillary controlled-release formulations.

**Key Words**—Controlled release, capillaries, volatile substances, pheromones, release rates, kinetics, predictive model.

## INTRODUCTION

The use of behavior-modifying chemicals in insect control strategies requires that they be disseminated from controlled-release formulations. Capillaries made of glass, polyester terephthalate, and Celcon® have been employed for some time as controlled-release vehicles for insect pheromones (Roelofs, 1979, and references therein). Such pheromone-containing capillaries have been used as trap baits in both monitoring (e.g., Haworth et al., 1982) and mass trapping programs (Pitman, 1971) as well as in control programs employing aerially applied



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pheromone to disrupt mating communication (Brooks et al., 1977; Doane et al., 1982 Golub et al., 1983).

The ideal controlled-release device utilizes all of the active material in a given time period, releasing it at a constant rate (zero-order kinetics). In the practical application of insect pheromones, it is unlikely that the ideal device will be achieved. As reported by Brooks (1980), after an initial burst, the release from capillaries remains relatively constant (pseudo zero-order kinetics); hence they would appear to be superior to impregnated plastic and rubber matrices, plastic laminates, microcapsules etc., all of which have first-order kinetics.

Richardson (1959), investigated the evaporation of liquids from tubes, of 4, 6, and 8 mm in diameter, into a gas stream and applied Stefan's law relating the rate of evaporation to the height of the vapor–air column above the surface of the liquid. He suggested that in tubes of too small a diameter the velocity profile of the diffusing vapor would be affected and the relationship would not hold. This statement, together with problems of erratic release rates during the field use of Albany International hollow fibers, both normal Celcon (white) and black Celcon used in disruption formulations (Weatherston, unpublished), and polyester terephthalate used in trapping studies (J. Sharpe, personal communication), prompted us to reexamine the release of volatile materials from capillaries with regard to (1) kinetics of the release, (2) the effect of the vapor–air column above the liquid, and (3) developing a predictive model for the release of volatile materials.

## METHODS AND MATERIALS

The compounds used in this study were obtained as follows: hexane, ethyl acetate, methanol, and *n*-butanol, distilled in glass, from Burdick and Jackson Laboratories Inc., Muskegon, Michigan; isopropyl alcohol and 1,2-dichloroethane from Fisher Scientific Co., Fairlawn, New Jersey; benzene from J.T. Baker Chemical Co., Phillipsburg, New Jersey; butyl acetate (gold star) and hexyl acetate from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin. All compounds were used without further purification.

The 0.0475-cm-radius glass capillaries were Kimax-51 capillary tubes purchased from Ace Glass Inc., Vineland, New Jersey. The 0.0244-cm-radius glass capillaries were Drummond Microcaps purchased from Drummond Scientific Company, Broomhall, Pennsylvania, and flame sealed at one end.

The wide-bore capillaries ($N$ = 5 or 7) were filled from a 25-$\mu$l Hamilton syringe to the desired level. The narrow-bore capillaries ($N$ = 7) were filled from a 10-$\mu$l syringe fitted with a fused silica needle of the type used for on-column injection in capillary gas chromatography. The tubes were put in a fume hood at 23–30°C with an average wind speed of 0.2 m/sec or in an environmental chamber at 15°C with an average wind velocity of 0.7 m/sec.

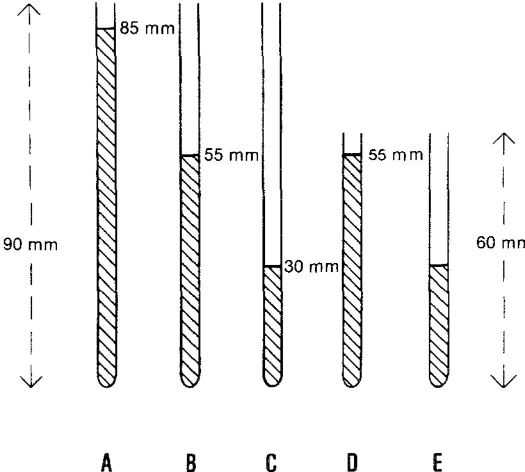


Fig. 1. Glass capillaries (0.0475 cm radius) used in the initial experiments

Weight-loss measurements were recorded on a Mettler M-S Microgramatic Balance (precision ±0.002 mg). For meniscus regression measurements, the evaporation was followed by direct reading from the open end of the capillary against 10 × 10 to the centimeter graph paper, or with a light microscope fitted with a reticle.

## RESULTS AND DISCUSSION

Initially, a series of five capillaries (0.0475 cm in radius) ($N$ = 5), set up as in Figure 1, were charged with hexane and the release at 30°C followed by weight-loss measurements. By plotting the amount released from the capillaries against time (Figure 2), it can be seen that capillaries A and D give similar curves, B and E give similar curves but release at a slower rate, and C releases at the slowest rate; that the rate is not zero order; and that the initial length of the vapor–air column is a critical factor in controlling the release rate. This can also be seen from Table 1 where the mean release rates over the initial 17.5 hr between A and D, and B and E are in good agreement.

Figure 3 illustrates the data for *n*-butanol released from 0.0475-cm-radius capillaries at 30°C. The release rates for benzene, isopropanol, and dichloroethane were also determined; the data comparing their release with that of hexane and *n*-butanol from A capillaries are shown in Figure 4.

The shape of the curves in Figures 2–4 suggest that the amount of material evaporating from the capillaries varies directly with the square root of time. This is borne out by regression analyses which give high $r^2$ values, e.g., for the

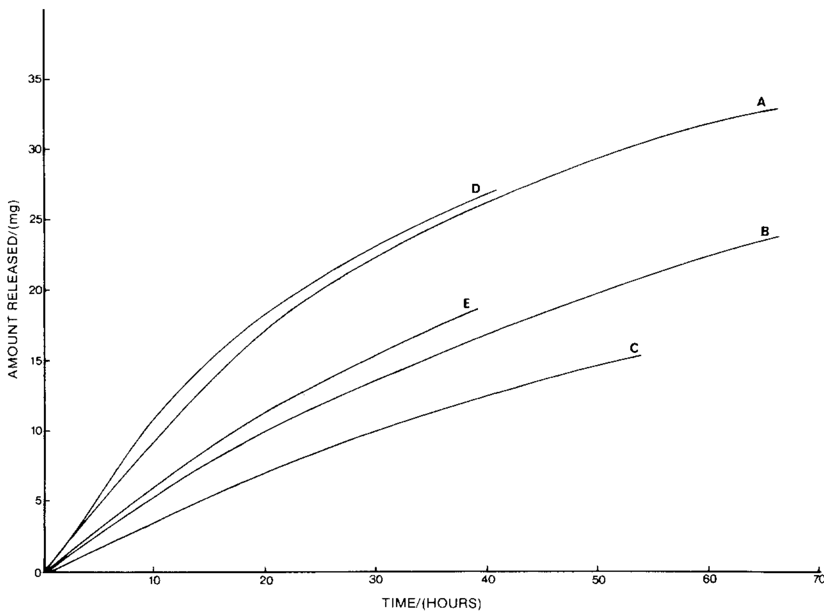


Fig. 2. Release of hexane from glass capillaries (0.0475 cm radius) as shown in Figure 1 at 30°C by the weight-loss method.

release of hexane (Figure 2): $r^2$ = 0.9994, 0.9994, 0.9992, 0.9990, and 0.9986, respectively, for capillaries A to E.

$$x = Kt^{1/2} \tag{1}$$

were $x$ = amount evaporated, $t$ = time, and $K$ = constant.

TABLE 1. MEAN RELEASE RATE ($\bar{X}$ ± SD) ($\mu$g/hr)/TIME PERIOD FOR HEXANE FROM GLASS CAPILLARIES (RADIUS = 0.0475 cm) AT 30°C BY WEIGHT LOSS

| | Time (hr) | | | | | | |
|---|---|---|---|---|---|---|---|
| Capillary | 17.5 | 23.9 | 29.3 | 40.3 | 46.8 | 52.5 | 63.9 |
| A | 909 (±33) | 508 (±59) | 478 (±40) | 343 (±13) | 329 (±15) | 327 (±15) | 288 (±9) |
| B | 521 (±13) | 409 (±11) | 377 (±7) | 296 (±6) | 294 (±6) | 287 (±5) | 266 (±6) |
| C | 359 (±12) | 318 (±11) | 314 (±6) | 246 (±8) | 240 (±5) | 196 (±6) | e |
| D | 970 (±27) | 533 (±11) | 486 (±11) | 371 (±12) | e[a] | e | e |
| E | 578 (±24) | 437 (±16) | 435 (±30) | e | e | e | e |

[a]e = capillaries empty.

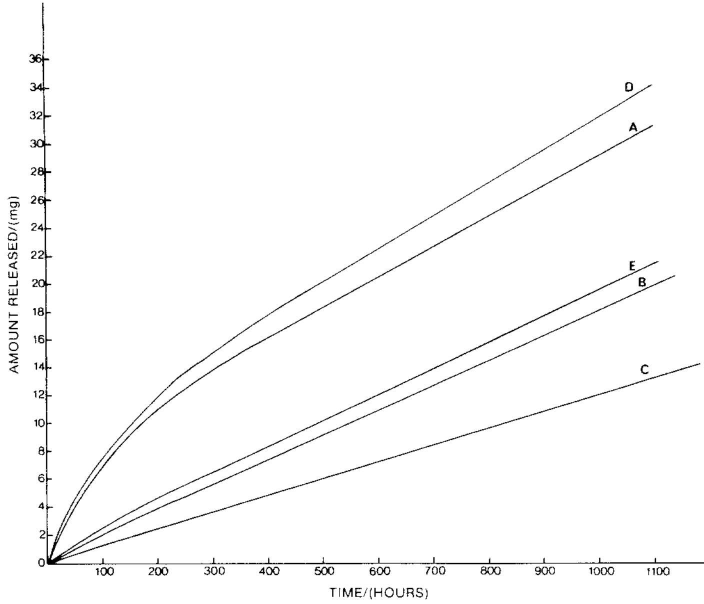


Fig. 3. Release of *n*-butanol from glass capillaries (0.0475 cm radius) as shown in Figure 1 at 25°C by the wight-loss method.

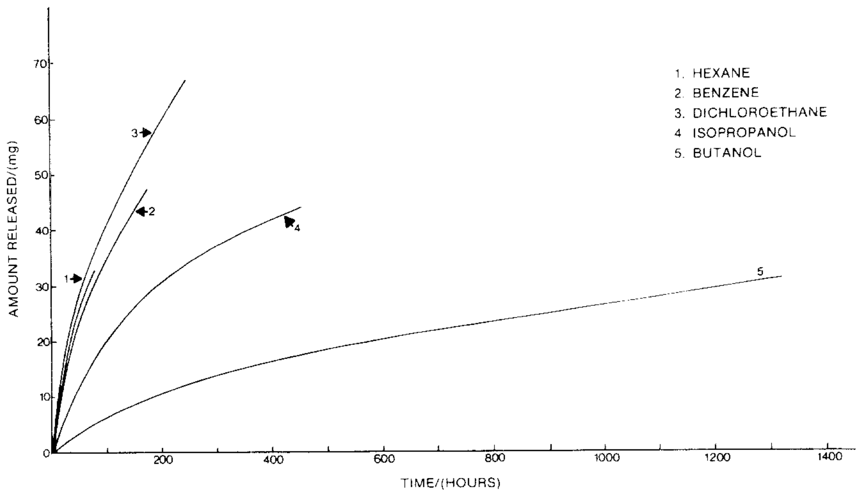


Fig. 4. Comparison of the release of five volatile compounds from A capillaries (0.0475 cm radius) at 25°C by meniscus-regression measurements.

From the volume of a cylinder, the amount evaporated can also be related to the length ($l$) of the vapor–air column within the capillary above the liquid surface

$$x = l \pi r^2 \rho \tag{2}$$

were $r$ = inner radius of the capillary, $\rho$ = density of the liquid charge.

Therefore,

$$\frac{dx}{dl} = \pi r^2 \rho \tag{3}$$

$$\frac{dx}{dt} = \frac{1}{2} K t^{-1/2} \tag{4}$$

Combining equations 3 and 4

$$\frac{dl}{dt} = \frac{K}{2 \pi r^2 \rho} \cdot t^{-1/2} \tag{5}$$

The mechanism of vapor release from capillaries, if transwall permeation is excluded, is, as reported by Ashare et al. (1975, 1976), Brooks et al., (1977) and Brooks (1980), a simple three-stage process: (1) evaporation at the liquid–vapor interface, (2) diffusion through the vapor–air column to the end of the capillary, and (3) convection away from the end of the capillary, with the diffusion step being the controlling factor. These authors developed an equation from a theoretical treatment of the release of volatile materials from capillaries relating rate with time

$$\frac{dl}{dt} = \left[\frac{-McD}{2\rho} \cdot \ln\left(1 - \frac{P_{vap}}{P}\right)\right]^{1/2} t^{-1/2} \tag{6}$$

where $M$ = molecular weight of the liquid, $c$ = molar density of the vapor–air column (Bird et al., 1960), $D$ = diffusion coefficient, $P_{vap}$ = vapor pressure of the liquid, $P$ = atomospheric pressure, and $\rho$ = density of the liquid.

Combining equations 5 and 6

$$K = \pi r^2 \left[- 2 McD\rho \ln\left(1 - \frac{P_{vap}}{P}\right)\right]^{1/2} \tag{7}$$

Table 2 shows the comparison of the calculated and experimentally derived values for $K$ for the five compounds studied and illustrates their agreement. This now permits us to calculate, instead of measuring experimentally, the rate of release for volatile compounds from glass capillaries

Substituting equation 2 into equation 1, and rearranging we obtain

$$t^{1/2} = \frac{l \pi r^2 \rho}{K} \tag{8}$$

TABLE 2. COMPARISON OF CALCULATED AND OBSERVED VALUES FOR $K$ FOR VARIOUS COMPOUNDS

| Compound | Amount ($\mu$g/hr$^{1/2}$) | | |
|---|---|---|---|
| | $K_{calc}$ | $K_{obsv}$ | $K_{calc}/K_{obsv}$ |
| Butanol | 805 | 862 | 0.934 |
| Isopropanol | 2000 | 2093 | 0.956 |
| Benzene | 3530 | 3782 | 0.933 |
| Hexane | 3776 | 4033 | 0.936 |
| 1,2-Dichloroethane | 4199 | 4479 | 0.937 |

Substituting equation 8 into equation 4 leads to

$$\frac{dx}{dt} = \frac{K^2}{2\pi r^2 \rho} \cdot \frac{1}{l}$$

which by substituting the value of $K$ (7) is equivalent to

$$\frac{dx}{dt} = -McD\pi r^2 \ln\left(1 - \frac{P_{vap}}{P}\right) \cdot \frac{1}{l} \tag{9}$$

Equation 9 is the basis of the model for predicting the release rate of volatile materials from glass capillaries which may be used to design capillary formulations. For practical considerations an algorithm was developed which allows us to calculate the release rate from the capillaries with respect to "$l$" and time, given the various constants and parameters, for the volatile material indicated in equation 9. In this way the effects of such factors as capillary length and radius, temperature, materials of different volatilities, and atmospheric pressure on the release rate may be tested very rapidly.

To test the model, the rates of evaporation of various compounds from glass capillaries of various lengths and of two internal diameters were measured at two temperatures. The validity of the model is shown in Figures 5–9.

Considering a situation where there is a requirement to release *n*-butanol at 10 $\mu$g/hr at 25° over 500 hr, it can be calculated from the results presented in Figure 3 with 0.0475-cm-radius capillaries that all five formulations would accomplish this. However, formulation C would be the most efficient of those illustrated. If the desired rate and longevity were 60 $\mu$g/hr over 115 hr, then only formulations A and D would give the required activity, with D being the more efficient since it uses less active material. However, in a case requiring the release of 60 $\mu$g/hr over 500 hr at 25°C, there are several choices, three of which are to use formulations A or D and reapply the treatment every 115 hr (i.e., 4.35 $\doteqdot$ 5 applications) or use a source consisting of six C capillaries. Simple calculations reveal that formulation A would require 2.36, and D 1.53 times

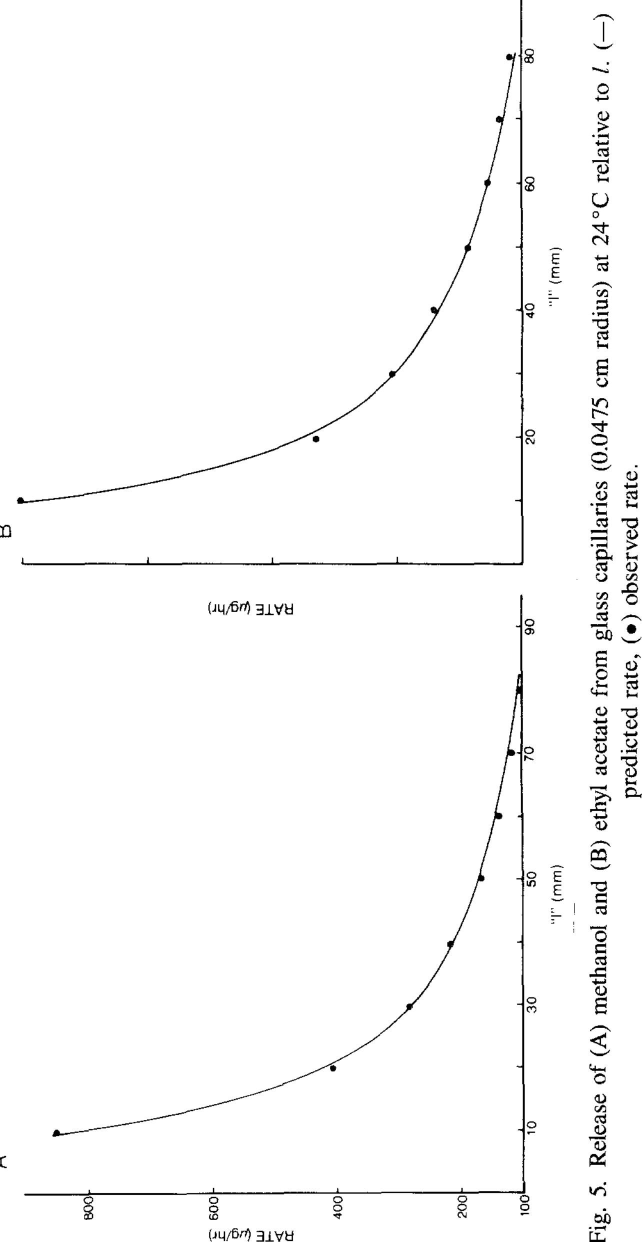


Fig. 5. Release of (A) methanol and (B) ethyl acetate from glass capillaries (0.0475 cm radius) at 24°C relative to *l*. (—) predicted rate, (●) observed rate.

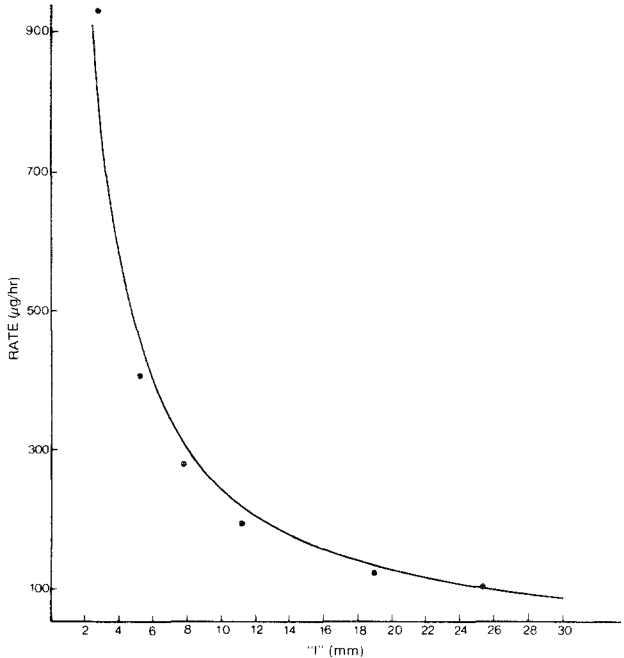


Fig. 6. Release of ethyl acetate from glass capillaries (0.0244 cm radius) at 23° relative to $l$. (—) predicted rate, (•) observed rate.

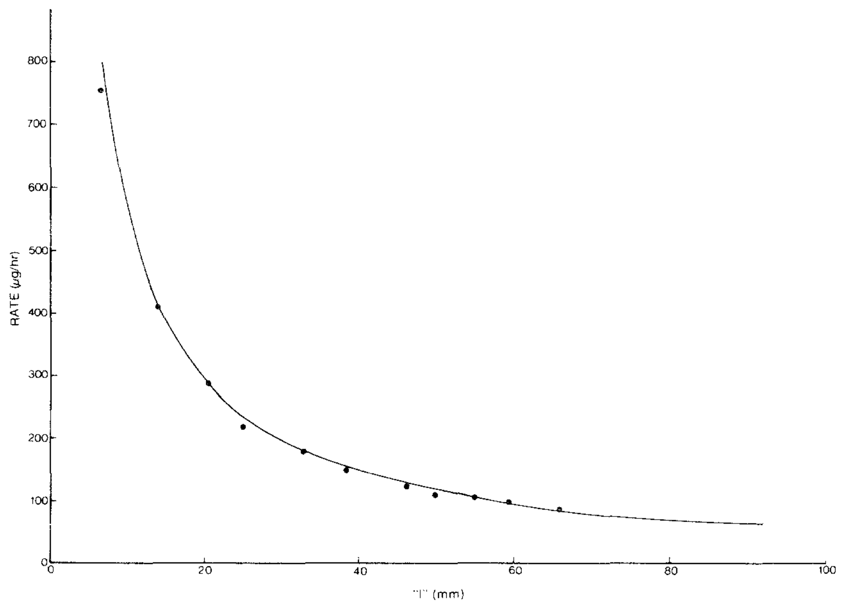


Fig. 7. Release of ethyl acetate from glass capillaries (0.0475 cm radius) at 15°C relative to $l$. (—) predicted rate, (•) observed rate.

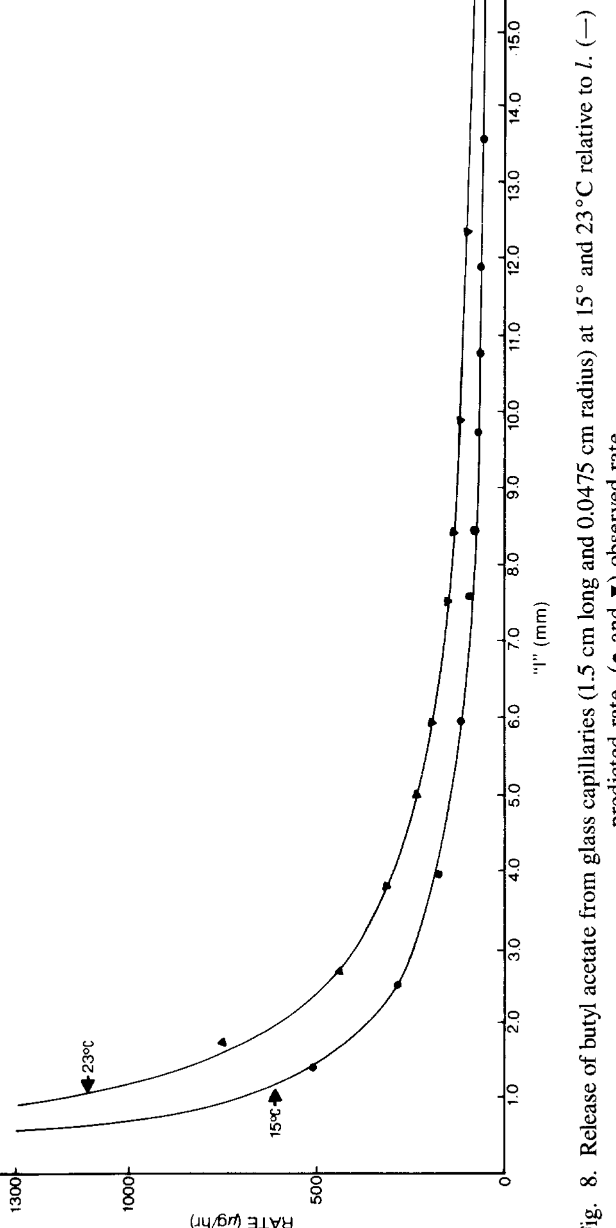


Fig. 8. Release of butyl acetate from glass capillaries (1.5 cm long and 0.0475 cm radius) at 15° and 23°C relative to *l*. (—) predicted rate, (● and ▼) observed rate.

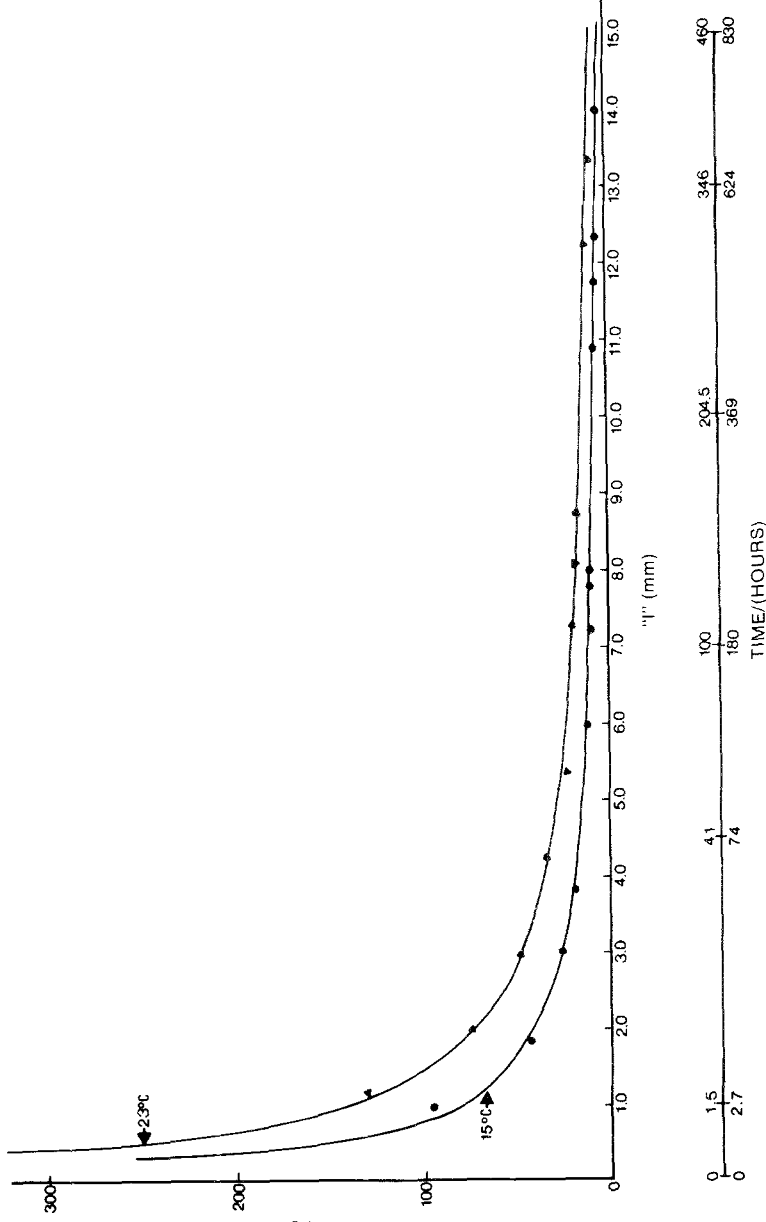


Fig. 9. Release of hexyl acetate from glass capillaries (1.5 cm long and 0.0475 cm radius) at 15° and 23°C relative to *l* and time. (—) predicted rate, (● and ▼) observed rate. Time scale upper numbers for 23°C, lower numbers for 15°C.

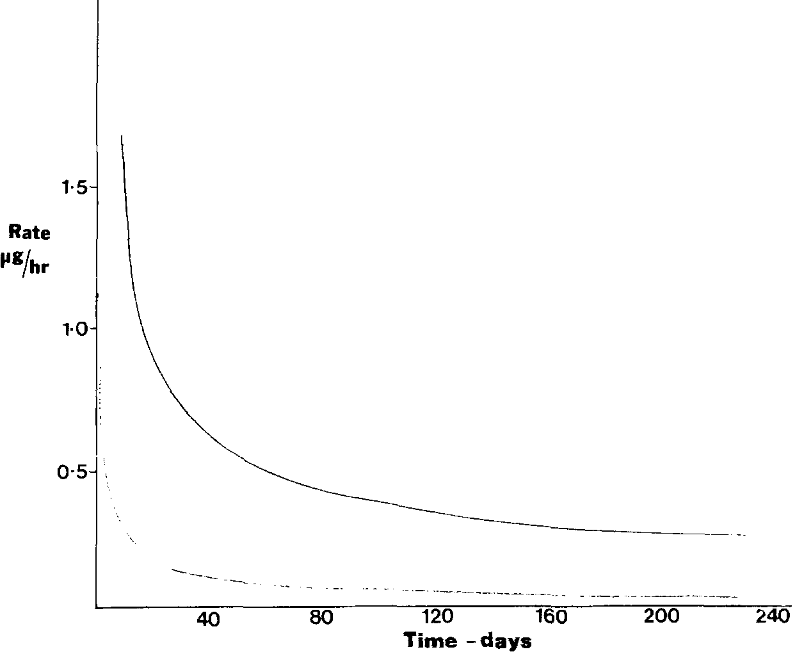


Fig. 10. Predicted release rate for (Z)-9-tetradecen-1-yl acetate from glass capillaries at 30°C. Upper curve, 1.5-cm-long × 0.0254-cm-radius capillaries; lower curve, 1.5-cm-long × 0.0102-cm-radius capillaries.

more active ingredient than the combination C formulation. Transferring this rationale to insect pheromones and their use for monitoring or control by aerial dissemination, when the current manufacturer-quoted price for bulk quantities of Z-9-tetradecen-1-yl acetate is (US) $4000.00/kg and for gossyplure is (US) $2000.00/kg, it is clear there is an urgent need to design better formulations with respect to both their efficacy and economics. We believe the development of predictive models will partly satisfy this need.

Figure 10 is the graphical representation of the prediction for the release of (Z)-9-tetradecen-1-yl acetate from both 0.0254-cm and 0.0102-cm-radius glass capillaries 1.5 cm in length at 30°C. The graph represents the release characteristics when the initial column of liquid in the capillaries is 1.45 cm in length. The device will take 220 days to empty, with, after the first 40 days, a mean release rate of 57.3 (± 13.6) ng/hr for the smaller-diameter capillary. If, in a given field situation, the requirement was for the release of this pheromone at 50 ng/hr for 45 days at 30°C, the model indicates that this may be obtained by using a 0.0102-cm-radius capillary of total active length 1.35 cm with an initial vapor–air column of 1.1 cm; this will ensure that the device will release at the correct rate of 50 ± 3 ng/hr from day 1 in the field and function over the

required 45 days. With the same longevity and temperature parameters but a release rate of 300 ng/hr for the same pheromone, the model indicates that a 0.0254-cm-radius capillary with the same fill characteristics as above should be used.

Work is in progress to further develop the model for predicting the release rates of binary mixtures, and to take into account the variations in temperatures found in field situations.

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# CAPILLARIES AS CONTROLLED RELEASE DEVICES FOR INSECT PHEROMONES AND OTHER VOLATILE SUBSTANCES—A REEVALUATION

## Part II. Predicting Release Rates from Celcon and Teflon Capillaries[1]

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(Received June 25, 1984; accepted October 24, 1984)

**Abstract**—A predictive model developed for the release rates of volatile materials from glass capillaries was invalid when tested with Celcon fibers used commercially in pheromone-based insect control strategies. Several factors which might explain the differences between the predicted and observed rates are discussed, and it is hypothesized that the topography of the fiber lumen is the major factor causing the observed rates to deviate from the predicted values and the large variation in rate from fiber to fiber. This hypothesis was tested using Teflon capillary fibers with both smooth and rough lumen walls and shown to be valid. This indicates that commercial hollow-fiber pheromone formulations can be improved both in efficiency and cost by careful selection of fiber material, improvement in fiber manufacturing, and the use of a predictive model in formulation design.

**Key Words**—Controlled release, capillaries, hollow fibers, pheromones, release rates, predictive model.

### INTRODUCTION

In a prevoius publication (Weatherston et al., 1984), we reported the development of a model for predicting release rates of volatile materials from glass capillaries. By means of an algorithm, the rate with respect to time and the length of the vapor–air column above the liquid charge may be calculated; in

[1]Contribution No. 391 Department of Biology, Laval University.



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this manner, the effects of such factors as capillary length and radius, temperature, atmospheric pressure, and materials of different volatilities may be tested.

Practical considerations obviate the widespread use of glass capillaries as pheromone controlled-release dispensers in the commercial arena; hence the utility of the model depends on its validity when tested with capillaries made of polymers such as are currently being, or may be, used in pheromone control strategies against insect pests. We now wish to report our findings when the model is applied to capillaries made of Celcon and Teflon.

## METHODS AND MATERIALS

The Celcon fibers were obtained from the Controlled Release Division of Albany International Company, Needham Heights, Massachusetts, and are of the types used commercially in their aerial broadcast insect control products. Teflon microtubing was purchased from Cole-Parmer Instrument Company, Chicago, Illinois, cut into the required lengths, and one end sealed with epoxy glue.

Glass capillaries, the volatile compounds tested, and the methodologies used were as previously described (Weatherston et al., 1984).

For scanning electron microscopy, longitudinal halves of Celcon and Teflon fibers, and pieces of glass capillaries were mounted on stubs with conductive paint, coated with a 500 Å layer of gold and the interior wall surface examined, under 15 kV, with a Cambridge S-150 scanning electron microscope.

## RESULTS AND DISCUSSION

The predictive model (Weatherston et al., 1984) relating release rates of volatile materials from glass capillaries to the length of the vapor–air column above the liquid appeared to be invalid when tested with 8 mil (0.0204 cm) diameter Celcon fibers such as those currently employed in the Albany International product Nomate-PBW for control of the pink bollworm on cotton. From Figure 1 it can be seen that the release of methanol from such fibers is not only in poor agreement with the predicted release rate, but that the rate variation from fiber to fiber is very large, most fibers releasing at a faster rate than predicted.

Some factors that might explain these differences between the predicted and observed release rates and the large interfiber variation include (1) absorption of the liquid charge into the fiber wall, (2) the effect on vapor pressure of the meniscus shape, which is dependent on the fiber material, (3) irregular diameter of the lumen along the length of the fiber, and (4) the topography of the lumen walls.

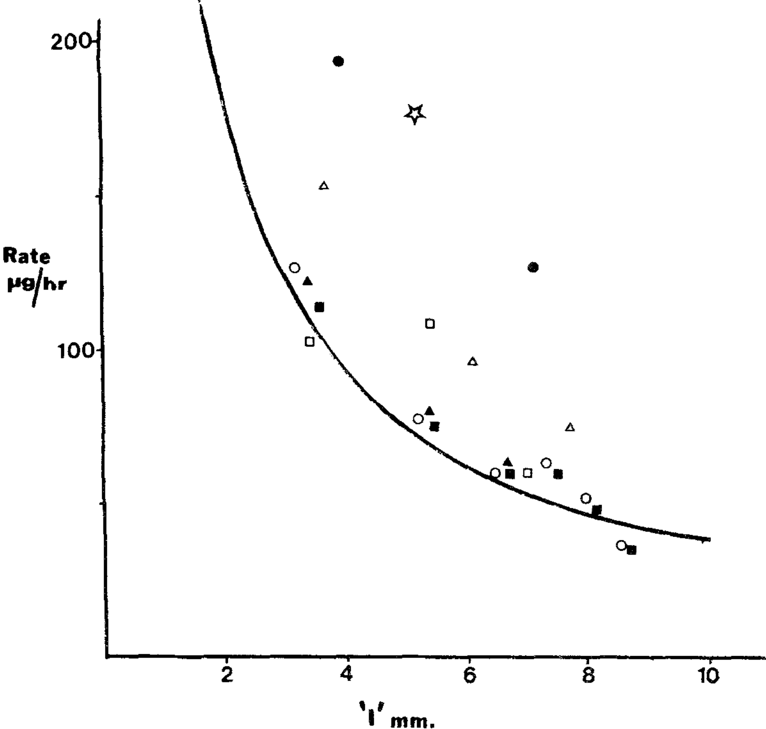



FIG. 1. Release of methanol from 1.5-cm-long Celcon fibers (8 mil diameter) at 23°C measured by miniscus regression. (—) = predicted rate, (other symbols) = observed rate for individual fibers.

Celcon (a polyoxymethylene–cooxyethylene copolymer) was selected by Albany International during the pioneering development work of hollow-fiber controlled-release systems, one reason being the high degree of crystallinity of this polymer, which would indicate that little or no absorption of the liquid charge into the walls would take place.

As reported by Brooks (1980), it is known from classical physical chemistry that the vapor pressure above a liquid is dependent on the radius of curvature of the surface. For different liquid–fiber material combinations the meniscus–fiber contact angles will vary, as will the radii of curvatures. However, we do not believe that this effect and that of absorption would cause the discrepencies between the predicted and measured release rates or be the cause of the large fiber–fiber variations observed.

The diameter of the lumen at various points along the active length (1.23 cm) was measured for 20 mil (0.0508 cm) diameter fibers ($N$ = 10). The mean values for randomly selected fibers were 0.0496 (±0.002) cm at the open end,

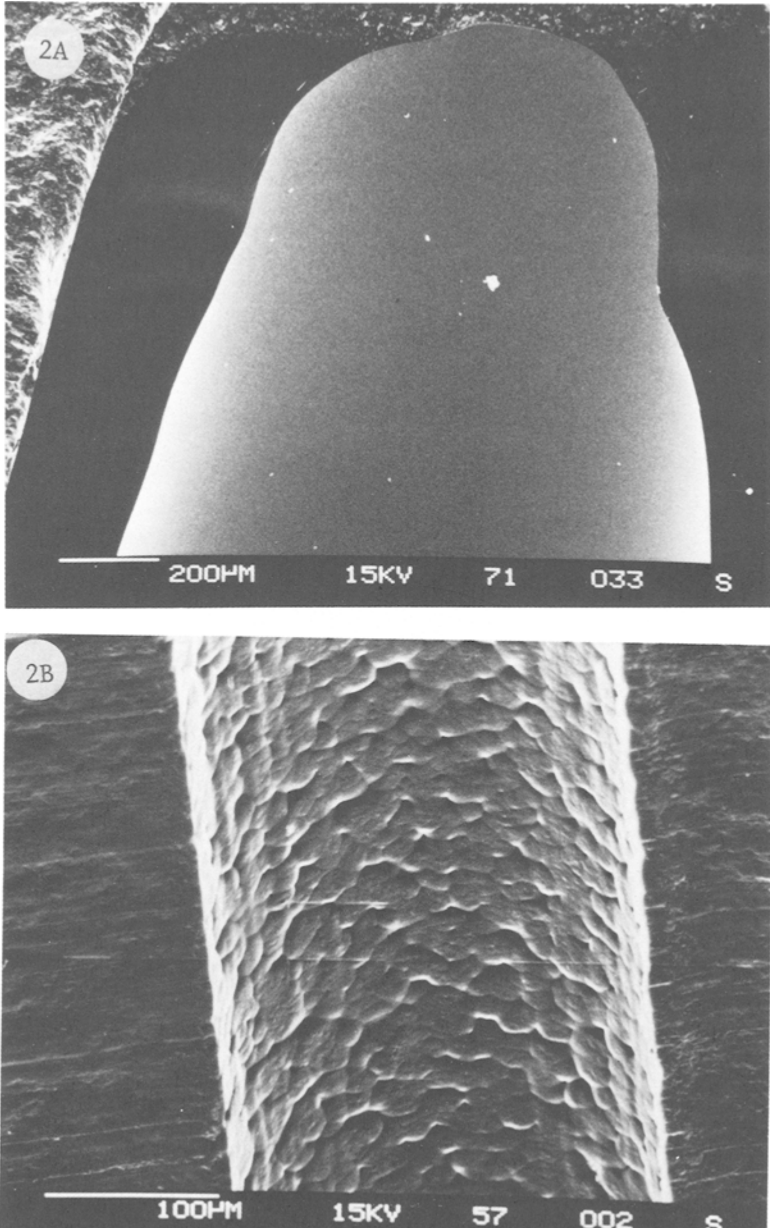
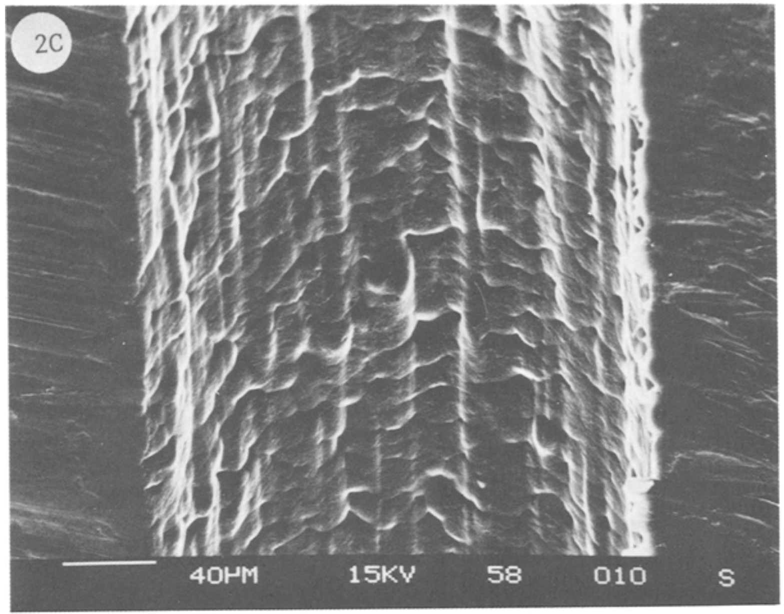



FIG. 2. Scanning micrographs of the lumen wall of capillaries: (A) glass capillary 0.095 cm diameter; (B)–(D) three individual 8-mil Celcon fibers which released hexyl alcohol at different rates.



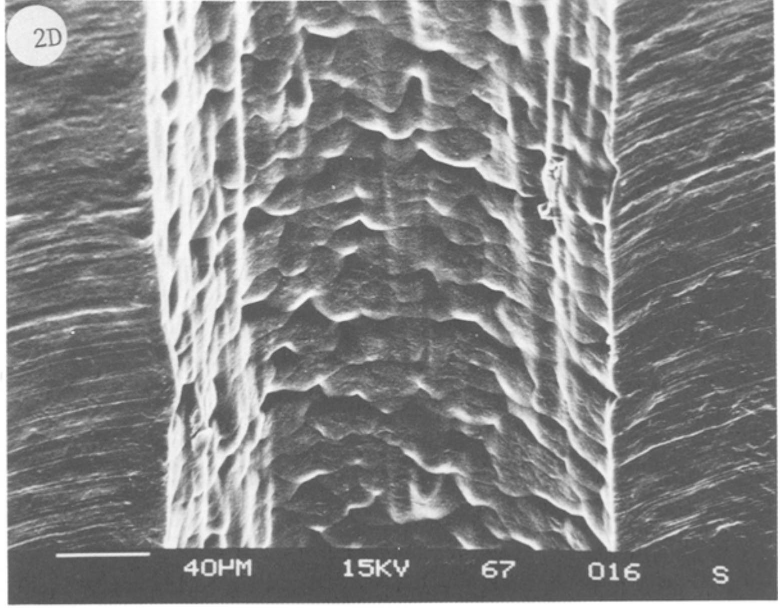




FIG. 2. Continued.

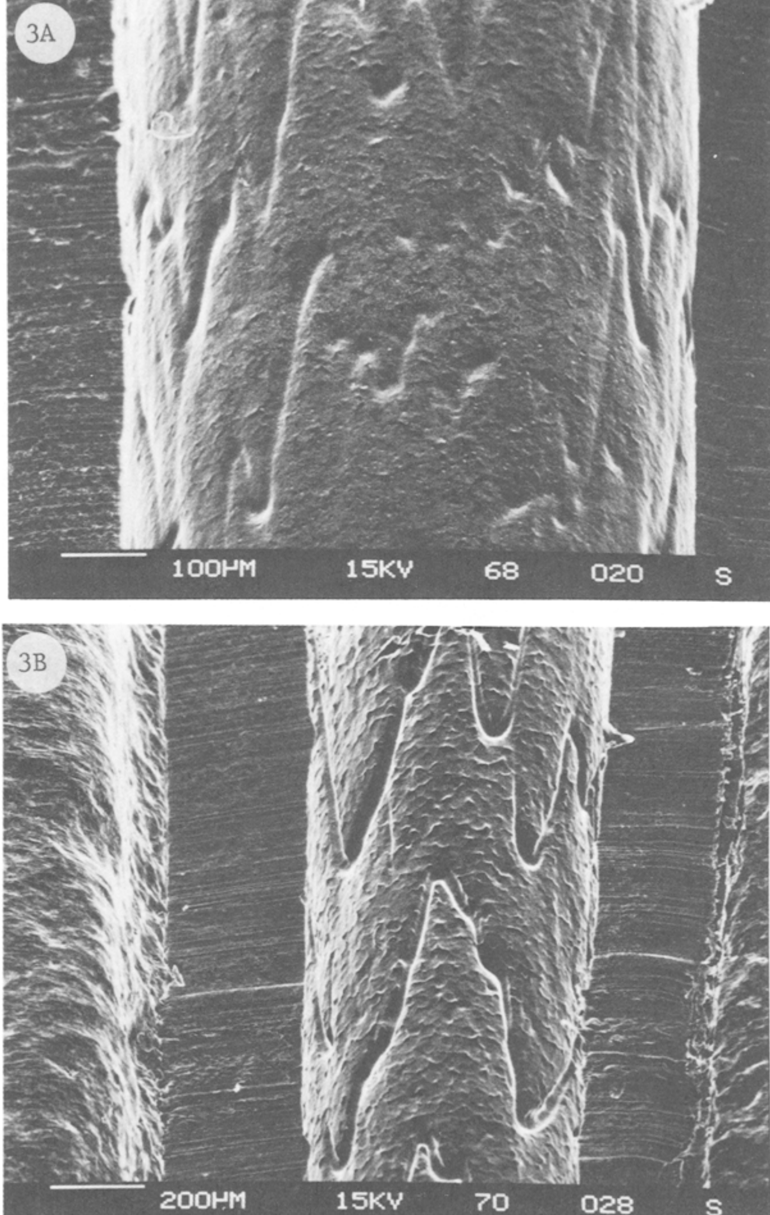



FIG. 3. Scanning electron micrographs of the lumen wall of 20-mil Celcon fibers. (A) Fiber exhibiting the slowest release of the three for hexyl acetate at 23.6°C; (B) fiber exhibiting a release rate intermediate to those shown in (A) and (C); (C) fiber exhibiting the fastest release rate.

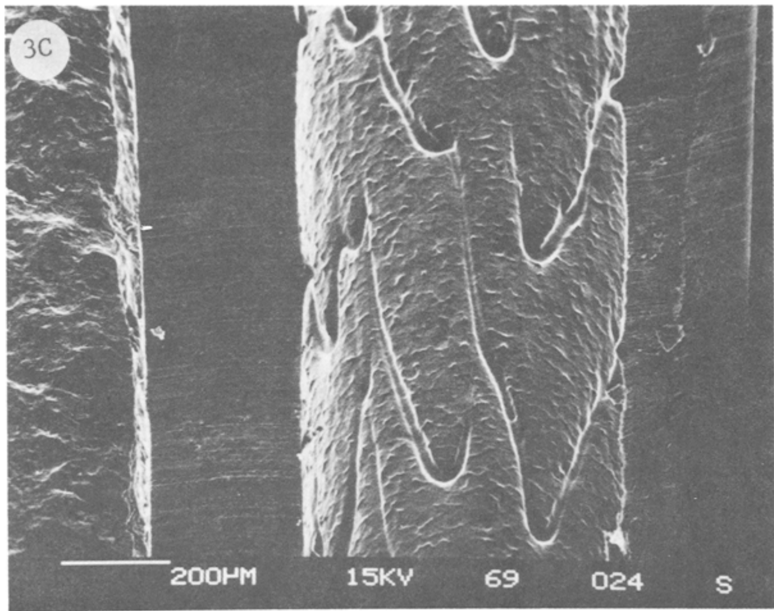



FIG. 3. Continued.

and 0.0503 (±0.001), 0.0504 (±0.002), and 0.0504 (±0.002) cm at 3, 6, and 9 mm respectively, from the open end. Once again this would not account for the differences between predicted and observed release rates or explain interfiber variations.

Scanning electron microscopy of the internal surface of many Celcon fibers and a comparison with the internal surface of glass capillaries revealed what we believe to be the major contributing factor to the erratic release of volatile materials from Celcon fibers. Figure 2A shows the smooth internal surface of the glass capillaries while Figure 2B–D illustrates the varying interfiber topography of the lumen walls of 8 mil (0.0204 cm) diameter Celcon fibers and shows the interstitial nature of the internal surface. The less smooth the lumen wall the greater the release rate of the volatile material since the receding liquid can be trapped in the crevices producing added release reservoirs. The predicted rate of hexyl alcohol from 8 mil capillaries at 23.6°C at $l$ (length of vapor–air column above the liquid surface) = 2.7 mm is 1.40 µg/hr; this may be compared to a rate of 1.48 µg/hr at $l$ = 2.7 mm for the fiber shown in Figure 2B, 7.82 µg/hr at $l$ = 2.7 mm, and 12.07 µg/hr at $l$ = 2.85 mm for the fibers shown in Figure 2C and D, respectively. Similar results were obtained with 20 mil (0.0508 cm) diameter Celcon fibers where, of the three fibers shown in Figure 3, the one in Figure 3A was the slowest releaser, that in Figure 3C the fastest releaser, with

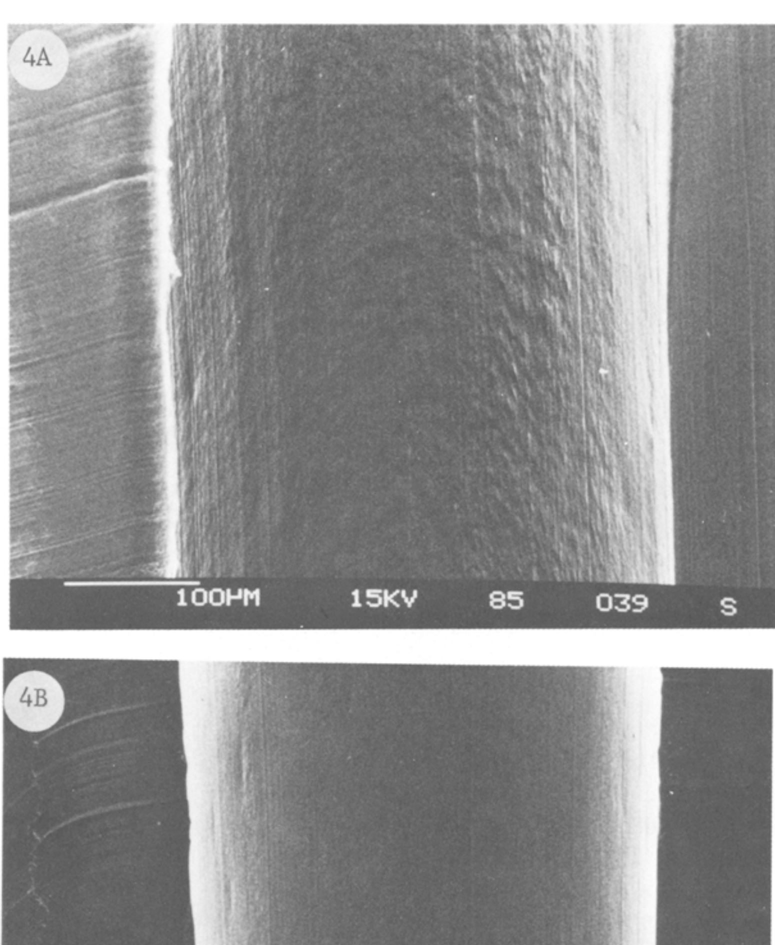



FIG. 4. Scanning electron micrographs of the lumen wall of microbore Teflon tubing: (A) unscratched "12-mil" capillary, (B) unscratched "32-mil" capillary, (C) scratched "32-mil" capillary.

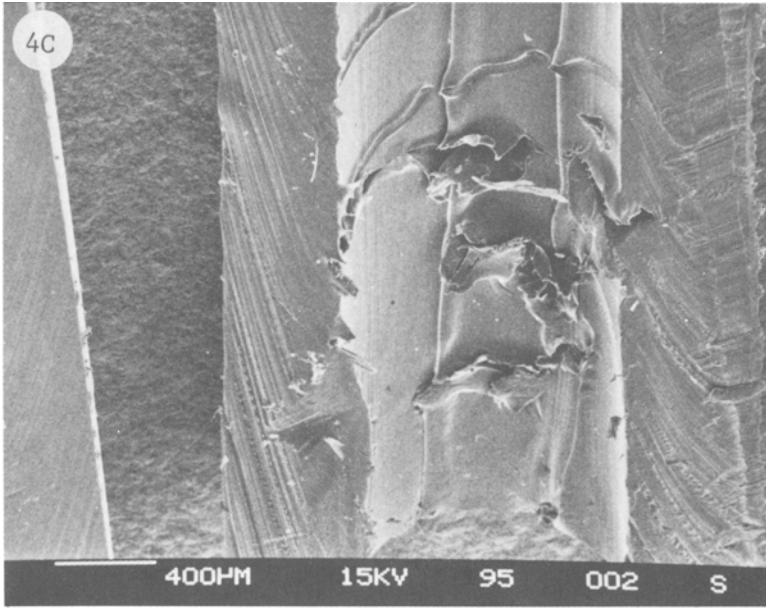



FIG. 4. Continued.

the fiber shown in Figure 3B having an intermediate release rate for hexyl acetate at 23.6°C.

To test the hypothesis that the roughness of the lumen walls was the major contributing factor for the poor agreement between predicted and observed release rates, we tested microbore Teflon capillaries as release devices. The micrographs (Figure 4A and B) show the smooth lumen wall of "12 mil" (0.0305 cm) diameter (actually 0.0326 cm) and "32 mil" (0.0812 cm) diameter (actually 0.0842 cm) Teflon capillaries, respectively, while Figure 5 presents the plot of the release rate versus $l$ at 23.6°C for both "12 mil" and "32 mil" Teflon fibers, 1.5 cm in length, charged with butyl acetate indicating the validity of the model. The small standard deviations indicate the small variation in release rate between individual fibers.

To further test our hypothesis, the internal surface of some "32 mil" Teflon fibers were scored with an insect pin (Figure 4C) and the release rate measured at 23.7°C relative to $l$ for seven individual fibers for seven time periods. Table 1 compares the mean release rate from unscratched Teflon fibers ($N = 7$) with that from seven individual scratched fibers and the mean rate of these seven fibers. The data show that the values for the release rate from the individual scratched Teflon fibers are not in good agreement with the values obtained from the unscratched fibers. The results also show that the mean rates for the unscratched Teflon fibers, which are in good agreement with predicted values,

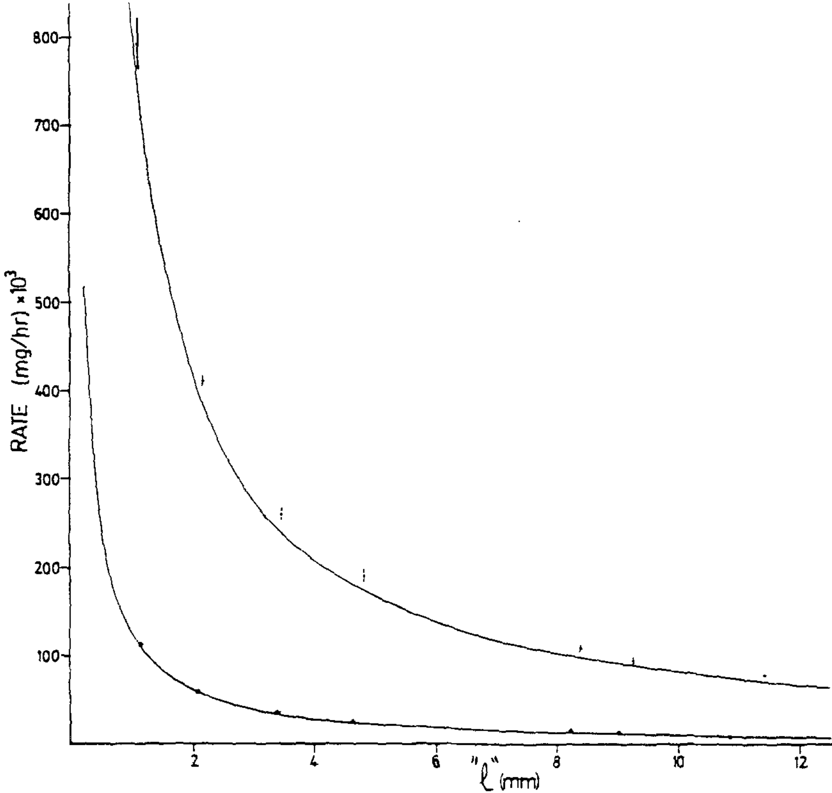



FIG. 5. Release of butyl cetate from unscratched Teflon capillaries, 1.5 cm in length, at 23.6°C. Upper trace, 0.0842-cm-diameter capillaries, lower trace, 0.0326-cm-diameter capillaries. (—) = predicted release, (•) = observed values. Vertical bars are the standard deviation.

have small standard deviations, while the mean rates from scratched fibers have large standard deviations, reflecting the large interfiber variation similar to that seen with the commercial Celcon fibers.

In summary, the predictive model developed for the release of volatile materials from glass capillaries (Weatherston et al., 1984) is valid for the release of volatile materials from capillaries made from other substances provided that (1) the lumen wall is smooth, (2) the capillary diameter is uniform throughout its active length, and (3) there is little or no absorption of the liquid charge into the capillary wall. As to the effect of the meniscus shape, the liquid–fiber combinations which we have studied all exhibit a meniscus concave on the vapor side, resulting in a vapor pressure less than the true equilibrium value and caus-

TABLE 1. RELEASE OF BUTYL ACETATE FROM UNSCRATCHED AND SCRATCHED TEFLON CAPILLARIES, 0.0842 cm IN DIAMETER AND 1.5 cm IN LENGTH AT 23.7°C.

| Time period (hr) | Unscratched fibers, $\bar{X}_7$ release rate (μg/hr) | Scratched fibers release rate (μg/hr) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | $\bar{X}$(SD) |
| 0–0.2 | 1737 (±103) | 2258 | 2403 | 2294 | 2549 | 2476 | 2440 | 3532 | 2565 (±406) |
| 0.2–0.7 | 737 (±33) | 1544 | 1049 | 1573 | 1661 | 1136 | 1661 | 2797 | 1632 (±529) |
| 0.7–1.43 | 508 (±39) | 1217 | 1097 | 958 | 938 | 908 | 1596 | 2704 | 1345 (±597) |
| 1.43–2.1 | 380 (±74) | 1283 | 902 | 359 | 370 | 826 | 1413 | 1759 | 986 (±487) |
| 2.1–3.03 | 317 (±20) | 1190 | 830 | 666 | 313 | 869 | 666 | 854 | 770 (±247) |
| 3.03–19.17 | 157 (±1) | e[a] | e | 198 | 178 | e | 98 | e | 158 (±43) |
| 19.17–23.68 | 107 (±3) | e | e | 78 | 82 | e | 81 | e | 80 (±2) |

[a] e = fiber empty.

ing a decrease in the release rate from the capillary. Therefore this factor cannot account for the large positive deviation from the predicted values observed with Celcon and scratched Teflon fibers. The results of our studies indicate that the current commercial hollow-fiber formulations of insect pheromones are inefficient in their utilization of the pheromones and that the efficiency of pheromone utilization and hence economics could be optimized by the use of new polymeric fibers, improving the quality of the present Celcon fibers as regards to the smoothness of the lumen wall, and the application of the predictive model in formulation design.

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# ISOLATION AND PARTIAL CHARACTERIZATION OF PHYTOTOXIC COMPOUNDS FROM LANTANA (*Lantana camara* L.)[1]

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**Abstract**—Phytotoxic compounds obtained from methanol extracts of field-grown lantana (*Lantana camara* L.) were quantified in terms of their inhibition of ryegrass (*Lolium multiflorum* Lam.) seed germination and/or seedling growth. Subsequent partition of the aqueous fraction (derived from drying the MeOH extract in vacuo and redissolving in distilled water) at various pHs with solvents of differing polarity indicated the phytotoxic compounds were both polar and slightly acidic. Thin-layer chromatographic separation of the acidic butanol fraction in a solvent mixture of butanol, acetic acid, water (4 : 1 : 5, by volume) yielded an active fraction with an $R_f$ value of 0.89–1.0 that was inhibitory to both root and shoot growth of ryegrass. Two other fractions with $R_f$ values of 0.04–0.23 and 0.41–0.57 were inhibitory only to root but not shoot growth.

**Key Words**—Allelopathy, allelochemics, lantana, *Lantana camara*, ryegrass, *Lolium multiflorum*, TLC, osmotic potential, inhibition.

## INTRODUCTION

Despite significant advances in weed control technology, crop losses caused by weeds and cost of weed control in the United States alone are estimated at $14 billion annually (Worsham, 1982). The repeated use of herbicides can result in the selection of herbicide-resistant biotypes (Bandeen et al., 1982; Gressel et

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al., 1982). In addition, increased public awareness of the possible adverse effects of synthetic pesticides on the environment necessitates research on alternative weed control methods.

One area of innovative research is allelopathy and its application to agriculture (Putnam and Duke, 1978; Putnam and DeFrank, 1983). Allelopathy, the regulation of growth of one plant species by chemicals released from another, occurs widely in many plant communities and is believed to regulate species density and distribution (Rice, 1984). Isolation and identification of natural compounds that are implicated in allelopathy are receiving greater research effort because it is possible that related compounds may be synthesized that can produce similar desirable effects as pesticides. Several plant products have been proven useful in control of insects (Jacobson, 1975), and the potential exists for the development of many other natural insecticides, herbicides, and growth regulators.

Lantana, rapid-growing perennial woody shrub, is a serious weed in Florida citrus groves. Its proliferation and spread are attributed to a number of factors, including natural propagation, reduced use of hand-weeding, high tolerance to currently available herbicides, and decreased competition from other weeds (Phillips and Tucker, 1976). In an earlier paper, we reported phytotoxicity of lantana (Achhireddy and Singh, 1984) residues to the test species milkweed vine (*Morrenia odorata* Lindl.) and suggested the involvement of allelopathy. The ways in which the chemical inhibitors were released into the soil from lantana residues were also determined. Characteristics of the inhibitory compound(s) reponsible for inhibition of milkweed vine were not determined. Milkweed vine, also a troublesome weed, is spreading in Florida citrus groves (Phillips and Tucker, 1970). In our earlier studies involving the examination of allelopathic effects of lantana, we used milkweed vine as an indicator species because we did not observe milkweed vine infestation near lantana bushes, suggesting an allelopathic mechanism. Since phenolic compounds have been implicated in most allelopathy studies (Guenzi and McCalla, 1966; Liebl and Worsham, 1983; Putnam and Duke, 1978; Rice, 1984; Tang and Young, 1982), we examined for their presence in lantana and for their phytotoxicity against ryegrass as an indicator species.

## METHODS AND MATERIALS

*Methanol Extracts and their Inhibitory Potential.* Lantana shoots were collected from field-grown plants near the Citrus Research and Education Center, Lake Alfred, Florida. Leaves and stems were separated and 100 g fresh weight of each was blended with 500 ml MeOH in a Waring blender for 2 min. The extract was vacuum-filtered through a Buchner funnel using Whatman No. 1 filter paper. The final volume of the filtrate was adjusted to 1000 ml with MeOH.

Preliminary experiments were conducted to detect any differences in the inhibitory potential between lantana stem and leaf extracts on ryegrass germination and growth. Ryegrass was chosen for the present studies because of its fast growth, uniform germination, and sensitivity to lantana phytotoxic compounds. Furthermore, ryegrass has been widely used as an indicator species in herbicide bioassays (Santelmann, 1977). One- or 5-ml aliquots of the extracts were placed on a filter paper in a 9-cm Petri dish. Filter papers were dried in a hood at lab temperature. Ten ryegrass seeds were placed in each Petri dish, 5 ml deionized water was added, and the Petri dishes were placed in a growth chamber maintained at 25/20°C (day/night) with an 8-hr photoperiod of 100 $\mu E/m^2/sec$. There were three replicates for each concentration, deionized water, and MeOH (1 or 5 ml). Ryegrass (cultivar Annual) seeds used in these studies were obtained from Florida Seed and Feed, Ocala, Florida. The germination of ryegrass used in these studies was over 95% and maximum germination occurred within three days. A seed was considered germinated if the radical visibly protruded through the seed coat. Final germination percentage and the lengths of roots and shoots were measured on the fifth day.

*Effect of Lantana Residues (MeOH-Insoluble Fraction) on Ryegrass Seedling Growth.* Methanol-insoluble fractions of lantana stem and leaf were air-dried, and the dried samples were added to sterilized sand in various proportions (0.05, 1.0, 2.0 and 4.0%, w/w). One hundred grams of this residue–sand mixture was added to Petri dishes and tested for biological activity using ryegrass as the indicator species. Fifteen ryegrass seeds were placed in each dish, watered thoroughly, and incubated at 25°C (±2°C)/14-hr photoperiod with a light intensity of 100 $\mu E/m^2/sec$. The germination percentage and root and shoot lengths were measured after eight days.

*Preliminary Partitioning of MeOH Extracts with Various Solvents.* The MeOH extracts (10%, w/v) were dried in a rotary evaporator at 40°C. The resultant residue was dissolved in 100 ml distilled water. The pH of the solution was 6.5. Aqueous fraction was partitioned successively with hexane, methylene chloride, ethyl acetate, and butanol at pH 2 and 11. In these preliminary experiments, the aqueous fraction was not partitioned at neutral pH because, if the phytotoxins were neutral, the activity would be detected in both acidic and basic fractions. The separation scheme is summarized in Figure 1.

All fractions (acidic, basic, and the aqueous remainder obtained for each solvent used) were tested for inhibitory potential. Three milliliters of each fraction were placed on a filter paper held in a Petri dish. Solutions were dried under a hood at laboratory temperature. Ten ryegrass seeds were plced in each Petri dish, and 5 ml of deionized water was added. Petri dishes were placed in a growth chamber. There were three replicates for each fraction. Final germination percentages and seedling growth measurements were made on the fifth day.

Based on the bioassays (see Table 3), further experiments involving solvent

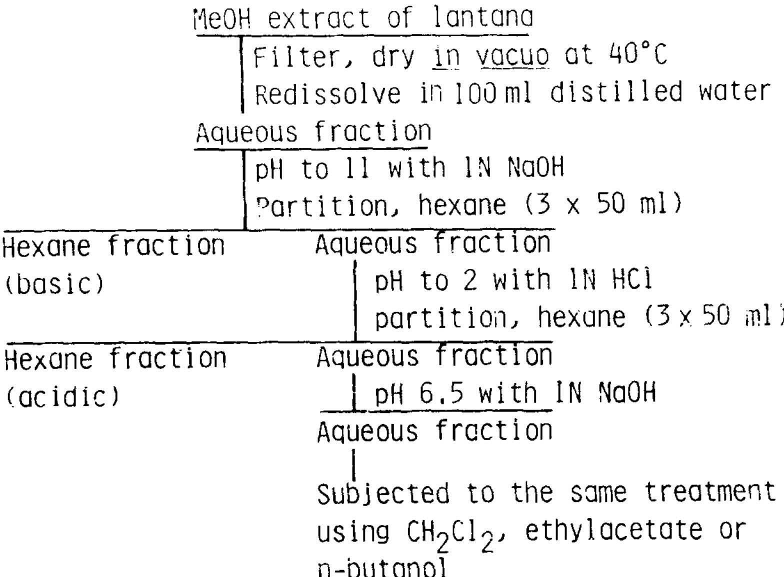


FIG. 1. Summary of procedures used to fractionate methanol extract of lantana foliage.

partition and TLC were carried out using the butanol fraction with some modifications.

*Butanol Partition and Thin-Layer Chromatography.* One hundred grams of fresh lantana leaves were ground in 1000 ml MeOH and filtered as previously described. The methanol extract was partitioned with petroleum ether to remove chlorophylls and the MeOH fraction was evaporated to dryness in vacuo at 40°C. The residue was taken up in 100 ml of distilled water. A 50-ml portion of this fraction was extracted at pH 2, 7, and 11 as outlined in Figure 2. Acidic and basic fractions were dried over anhydrous $MgSO_4$, filtered through a Buchner funnel with fritted disk (medium porosity, 0.15 $\mu$m), and the butanol fractions saved. All fractions [original aqueous, butanol (acidic, basic, and neutral), and the final aqueous remainder] were tested for inhibitory activity as described earlier.

The maximum inhibitor activity was present in the acidic fraction (see Table 5), and further separation using TLC methods were carried out on this fraction. One milliliter of the acidic fraction was separately streaked on a silica plate (Eastman chromatogram sheet; Eastman Kodak, Rochester, New York), and the plate was developed three times in a solvent mixture of butanol, acetic acid, and water (4 : 1 : 5, by volume, upper layer) known to separate phenolic compounds (Harborne, 1961). The plates were dried in the hood at lab temperature and examined under UV at 254 and 320 nm for absorption and fluorescence when sprayed with the Folin-Ciocalteau reagent (Krebs et al., 1969). All fluorescent

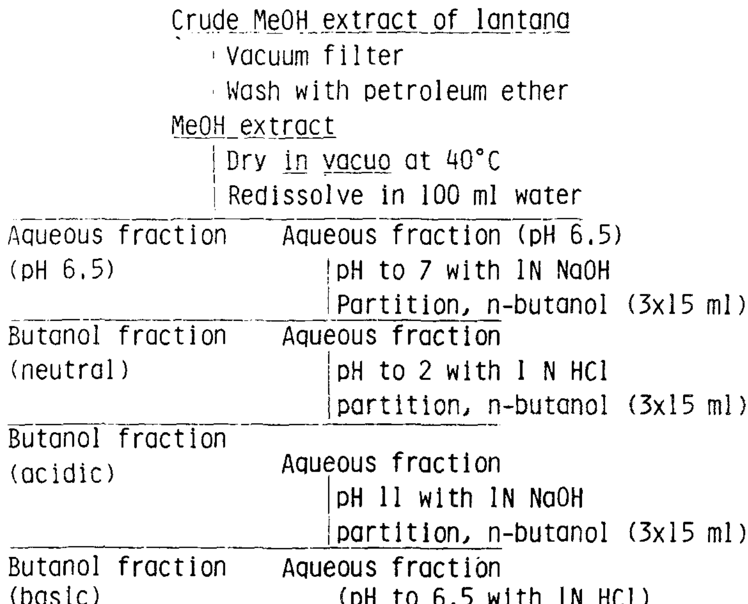


FIG. 2. Summary of procedures used to fractionate methanolic extract of lantana herbage using *n*-butanol.

or absorbent- and reagent-reactive bands were removed by scraping. Scrapings were eluted with 5 ml of distilled water. The silica coating and 5 ml of water were thoroughly mixed in a test tube, centrifuged in a clinical centrifuge and the supernatant used for bioassays. Thin-layer chromatographic plates developed in butanol–acetic acid–water mixture without the application of the extract were subjected to the same procedure and used as controls.

*Osmotic Potential Measurements.* Osmotic potentials of assay media were determined on a vapor-pressure osmometer (vapor-pressure osmometer model 5100B, Wescor Inc., Logan, Utah). In separate experiments, NaCl was added to distilled water to produce a water potential up to −130 kPa (comparable to those of lantana extracts), and the effect of this solution was also tested on the germination and growth of ryegrass.

*Analysis of Data.* All experiments were repeated, and the data were pooled for presentation. The results were analyzed by analysis of variance and the means were compared using least significant different (LSD) at the 5% level.

## RESULTS AND DISCUSSION

Crude MeOH extracts of lantana stem or leaf were inhibitory to ryegrass seedling growth, and the degree of inhibition was dependent on the concentra-

TABLE 1. EFFECT OF MeOH EXTRACTS OF LANTANA LEAF OR STEM ON RYEGRASS SEEDLING GROWTH AFTER 5 DAYS

| Treatments | Concentration (mg/ml)[a] | Growth (mm) | |
|---|---|---|---|
| | | Root | Shoot |
| Control, water | 0 | 52 | 34 |
| Control, MeOH | 0 | 44 | 33 |
| Leaf extract | 20 | 25[b] | 26[b] |
| Leaf extract | 100 | 2[b] | 10[b] |
| Stem extract | 20 | 35[b] | 32 |
| Stem extract | 100 | 10[b] | 19[b] |

[a]Based on fresh weight.
[b]Significantly different from control at 95% probability level as determined by LSD tests.

tion of the extract used (Table 1). The MeOH control had no significant effects on ryegrass seedling growth. Lantana extracts had greater inhibitory activity on ryegrass root growth than on shoot growth. Germination, however, was not affected by the MeOH extracts (data not shown).

Lantana leaf or stem residues (MeOH-insoluble fraction) were inhibitory at high concentrations (20 mg and 40 mg/g) to root but not to shoot growth (Table 2). There was, in fact, some enhancement in shoot growth by stem and leaf residues at 10–40 mg/g and 10–20 mg/g, respectively.

Butanol was the most effective partitioning solvent tested with regard to overall growth inhibition (Table 3). Although the ethyl acetate (acidic fraction) equally inhibited root growth, little or no growth inhibitory effects were observed on shoot growth.

TABLE 2. INFLUENCE OF LANTANA RESIDUES (MeOH INSOLUBLE) ON RYEGRASS SEEDLING GROWTH

| Residue concentration (mg/g) | Growth (mm) | | | |
|---|---|---|---|---|
| | Leaf residues | | Stem residues | |
| | Root | Shoot | Root | Shoot |
| 0 (control) | 54 | 54 | 54 | 55 |
| 5 | 48 | 64 | 60 | 63 |
| 10 | 51 | 76[a] | 58 | 77[a] |
| 20 | 41[a] | 75[a] | 48 | 83[a] |
| 40 | 30[a] | 64 | 30[a] | 90[a] |

[a]Significantly different from control at 95% probability level as determined by LSD tests.

TABLE 3. SOLVENT PARTITION OF PHYTOTOXINS PRESENT IN MeOH EXTRACTS[a]

| Solvent | Fraction | Germination (%) | Growth (mm) | |
|---|---|---|---|---|
| | | | Root | Shoot |
| | Control, water | 98 | 39 | 30 |
| Hexane | Control, hexane | 93 | 38 | 26 |
| | Acidic | 93 | 22[b] | 18[b] |
| | Basic | 100 | 34 | 18[b] |
| | Aq. remainder | 0[b] | —[b] | —[b] |
| $CH_2Cl_2$ | Control, $CH_2Cl_2$ | 80 | 37 | 31 |
| | Acidic | 100 | 10[b] | 26 |
| | Basic | 100 | 38 | 33 |
| | Aq. remainder | 90 | 3[b] | 8[b] |
| Ethyl acetate | Control, ethyl acetate | 100 | 40 | 30 |
| | Acidic | 100 | 7[b] | 26 |
| | Basic | 100 | 51[b] | 37 |
| | Aq. remainder | 0[b] | —[b] | —[b] |
| Butanol | Control, butanol | 100 | 37 | 29 |
| | Acidic | 95 | 8[b] | 16[b] |
| | Basic | 95 | 35 | 30 |
| | Aq. remainder | 80 | 9[b] | 13[b] |

[a]The extracts at pH 2 and 11 were fractionated successively with hexane, methylene chloride, ethyl acetate, and butanol.
[b]Significantly different from controls at 95% probability level as determined by LSD tests.

The butanol (acidic) fraction inhibited root and shoot growth by about 80 and 60% of the water control, respectively (Table 3). Therefore, the phytotoxins present in lantana appear to be primarily polar and slightly acidic. It was also interesting to note that even after partitioning with various solvents, significant inhibitory activity was still present in the aqueous remainder. These results suggested that lantana contain water-soluble phytotoxins. This is consistent with our earlier observation that water leachates of lantana incorporated into the potting soil were extremely inhibitory to milkweed vine seedling growth (Achhireddy and Singh, 1984).

In studies concerning the effects of the plant extracts on germination and growth of sensitive indicator species, osmotic potential of the solution can be a problem. Therefore, it is important to test for osmotic effects.

Osmotic potential of aqueous fractions, obtained by evaporation of solvent fractions and then redissolving in distilled water for biossays, ranged between −23 and −138 kPa (Tables 4 and 5). These values were too high to affect

TABLE 4. OSMOTIC POTENTIAL OF MeOH EXTRACTS VS. GROWTH INHIBITION OF RYEGRASS

| Extract volume (ml) | Final germination (%) | Solution osmotic potential (-kPa) | Growth (mm) | |
|---|---|---|---|---|
| | | | Root | Shoot |
| 0 (control) | 100 | 23 | 48 | 30 |
| 0.025 | 100 | 23 | 39[a] | 30 |
| 0.05 | 95 | 23 | 37[a] | 31 |
| 0.1 | 100 | 23 | 34[a] | 32 |
| 0.5 | 90 | 92[a] | 15[a] | 27 |
| 1.0 | 100 | 92[a] | 8[a] | 21[a] |
| 1.5 | 95 | 92[a] | 6[a] | 12[a] |
| 2.0 | 80[a] | 92[a] | 2[a] | 5[a] |

[a]Significantly different from control a 95% probability level as determined by LSD tests.

growth of ryegrass seedlings. Methanol extracts of 0.5–2.0 ml produced the same osmotic potential of −92 kPa of the bioassay medium (Table 4). However, less germination and greater growth reduction of ryegrass was observed in 2.0 ml than in 0.5 ml treatment. There were no significant differences in osmotic potential among the various volumes within each of the basic or neutral butanol fractions. Significant differences in percent germination and seedling growth were evident, however, between these volumes (Table 5). This suggests that inhibition resulted from the phytotoxins present in the extracts and not from osmotic stress. In addition, ryegrass seeds germinated and grown in a NaCl solution with osmotic potential equivalent to that of the extracts showed no significant reduction in percent germination or seedling growth (data not included).

A large portion of the phytotoxic substances present in the aqueous extract (following MeOH evaporation) partititioned into the butanol acidic and neutral fractions (Table 5). A 1-ml acidic fraction inhibited growth in length of ryegrass root and shoot by about 95 and 97%, respectively. A 1-ml neutral fraction inhibited root and shoot lengths by about 93 and 84%, respectively. The basic fraction reduced growth by about 50%. Although percent germination was not affected by the acidic and neutral fractions, seedling growth was completely inhibited. One milliliter of the aqueous remainder was also inhibitory, reducing root and shoot lengths by 65 and 40%, respectively.

Chromatographic analysis of the acidic butanol fraction revealed several bands (by UV and color reaction) (Table 6). The eluate from one band with an $R_f$ value of 0.89–1.0 inhibited growth of roots and shoots by 66 and 27%, respectively. Two other bands with $R_f$ values of 0.04–0.23 and 0.41–0.57 were also significantly inhibitory only to root but not shoot growth. Greater inhibitory action on root growth than on shoot growth in these chromatographically sepa-

TABLE 5. OSMOTIC POTENTIAL OF VARIOUS BUTANOL FRACTIONS OF LANTANA FOLIAGE VS. GROWTH INHIBITION OF RYEGRASS

| Fraction | Volume (ml) | Osmotic potential (–kPa) | Germination (%) | Growth (mm) | |
|---|---|---|---|---|---|
| | | | | Root | Shoot |
| Control, water | – | – | 95 | 43 | 31 |
| Acidic | 0.1 | 46 | 100 | 33 | 27 |
| | 1.0 | 46 | 70 | 2 | 1 |
| | 2.0 | 46 | 65 | 0 | 0 |
| Basic | 0.1 | 23 | 100 | 41 | 31 |
| | 1.0 | 23 | 100 | 19 | 14 |
| | 2.0 | 23 | 100 | 11 | 7 |
| Neutral | 0.1 | 23 | 95 | 29 | 28 |
| | 1.0 | 23 | 95 | 3 | 5 |
| | 2.0 | 23 | 35 | 0 | 0 |
| Aq. remainder | 0.1 | 46 | 100 | 43 | 28 |
| | 1.0 | 115 | 100 | 16 | 18 |
| | 2.0 | 138 | 100 | 13 | 17 |
| LSD (0.05) | | 14 | 25 | 5 | 5 |

TABLE 6. THIN-LAYER-CHROMATOGRAPHIC SEPARATION OF PHYTOTOXINS PRESENT IN BUTANOL (ACIDIC) FRACTION

| $R_f$ | Folin test | UV absorption (ab) or fluorescence (fl) | | Growth (mm) | |
|---|---|---|---|---|---|
| | | 254 nm | 320 nm | Root | Shoot |
| 0.0–0.04 | | | | 36 | 27 |
| 0.04–0.23 | | | | 29[a] | 27 |
| 0.23–0.26 | | | | 42 | 31 |
| 0.26–0.33 | + | ab | fl | 38 | 27 |
| 0.33–0.41 | | | | 37 | 30 |
| 0.41–0.57 | + | | ab | 24[a] | 25 |
| 0.57–0.63 | | | | 35 | 26 |
| 0.63–0.71 | + | ab | fl | 34 | 31 |
| 0.71–0.76 | | | | 37 | 29 |
| 0.76–0.82 | | | fl | 42 | 30 |
| 0.82–0.86 | | ab | | 36 | 31 |
| 0.86–0.89 | | ab | fl | 42 | 29 |
| 0.89–1.00 | + | ab | fl | 13[a] | 19[a] |
| Blank | | | | 38 | 26 |

[a]Significantly different from control at 95% probability level as determined by LSD tests.

rated phytotoxins is consistent with the experiments involving crude extracts (without TLC monitoring) (Tables 1, 3–5). On the basis of UV and color reaction data, the phytotoxic compounds present in lantana may be phenolic in nature. We are currently identifying these chromatographically separated phytotoxic compound(s) using HPLC and GC-MS methods.

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# SEX PHEROMONE OF TOBACCO STEM BORER, *Scrobipalpa heliopa* (LOWER) (LEPIDOPTERA: GELECHIIDAE)

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**Abstract**—The major volatile component in the extract of the female sex pheromone gland of *Scrobipalpa heliopa* was shown to be (*E*)-3-tridecen-1-ol acetate (V). The identification was based on mass spectral analyses, comparison of retention times with those of synthetic compounds on polar, nonpolar, and liquid crystal gas chromatographic columns and microchemical studies. The latter included hydrolysis and reacetylation, and mass spectral studies of the derivatives formed by epoxidation and methoxymercuration–demercuration. Analysis of gland extracts by gas chromatography linked to electroantennography showed this component to be the only one with significant biological activity, similar to that of the synthetic compound. (*E*)-3-Tridecenyl acetate (V) attracted male moths to traps in the field while addition of the *Z* isomer (III) reduced the numbers of moths caught.

**Key Words**—Tobacco stem borer, *Scrobipalpa heliopa*, sex pheromone, Lepidoptera, Gelechiidae, (*E*)-3-tridecen-1-ol acetate.

## INTRODUCTION

The tobacco stem borer, *Scrobipalpa heliopa* (Lower) [*Gnorimoschema heliopa* (Lower)] is a serious pest of tobacco in India (Patel and Chari, 1977), and of cultivated and wild tobacco and eggplants in parts of Africa, Australia, and regions of Asia (Anon., 1977). Control measures have been directed towards killing the eggs or first-instar larvae, since the later stages of development occur inside the plant tissue. The sex pheromone would provide a useful means of



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monitoring the population density of adult males as part of an integrated pest-management system.

## METHODS AND MATERIALS

*Insects.* A number of pupae were received from India periodically between 1981 and 1983. A batch of pupae was kept in a cage in a controlled environment room, maintained at 24–26°C, 70–80% relative humidity, and a 12-hr light cycle (12:12 hr scotophase/photophase). The pupae were kept moist and emerged adults were sexed and segregated into separate cages on the first day after emergence.

*Collection of Pheromone.* Female moths (3–4 days old) were collected at midscotophase, placed in a refrigerator (0°C) for 10 min, and then frozen on a piece of solid $CO_2$. The terminal 3–4 abdominal segments were dissected and then extracted with methylene chloride (500 μl). The extract was passed through a small column of Florisil and then concentrated to a volume of 75 μl by passage of a stream of dry, oxygen-free nitrogen.

*Chromatography.* Gas–liquid chromatography (GC) was conducted using Pye 104 and series D chromatographs (Pye-Unicam, Cambridge). The following gas chromatography analytical columns were used: (A) 3.1-m × 2-mm glass column containing 5% polyethylene oxide (Carbowax 20 M) on 100–120 mesh Diatomite Q, acid–alkali washed, DMCS treated; (B) same as (A) except the liquid phase was 5% FFAP; (C) same as (A) except the stationary phase was 5% OV-101 on Diatomite CLQ; (D) 6.2-m × 2-mm glass column containing 10% OV-275 on 100–120 mesh Diatomite CLQ; (E) 1.8-m × 2-mm glass column containing a smectic liquid crystal phase (Lester, 1978), 10.2% diethyl 4,4′-azoxydicinnamate, on 100–120 mesh Gas Chrom Q; (F) 50-m × 0.2-mm SCOT glass capillary column coated with Carbowax 20 M, using an injector similar to that described by Grob (Grob and Grob, 1978), but without a septum purge; (G) same as (A) except the liquid phase was 5% OV-275 on 100–120 mesh Diatomite CLQ.

*Electroantennography (EAG).* EAG measurements were obtained using whole insect preparations. The insects were anaesthetized ($CO_2$) and mounted with the antennae spread out, ventral surface uppermost. Glass electrodes drawn out of capillary tubing (Medelec GC-100 F-4) were filled with insect Ringer (Minks et al., 1974) and connected to a high-impedance ($10^{12}$ Ω) differential amplifier (gain × 100) via chloridized silver wires inserted down the center of each electrode. The microelectrodes were inserted into the antennae of the insect, the "indifferent electrode" in the base of one antenna and the "active electrode" in the tip of the other antenna, with the aid of micromanipulators and a binocular microscope.

The sample to be tested was placed in a Pasteur pipet and, after allowing the solvent to evaporate (1 min), puffed from a fixed distance (2 cm) over the antennae using purified air (cylinder, BOC) via a solenoid-controlled valve. As a control, the same procedure was performed without the test solution (i.e., solvent only).

EAG was also used to monitor the effluent from a GC column (GC-EAG), allowing correlation of EAG activity with peaks detected by a standard flame ionization detector (FID), fitted to a modified Pye 104 GC. The effluent from a capillary column (column F, 80°C then 6°C/min to 200°C) was split between the FID and a small glass reservoir (2 $cm^3$) (ratio ca. 1:4). Helium (4 $cm^3$) was injected into the reservoir at 10-sec intervals, sweeping the collected column effluent into a stream of air (500 $cm^3$/min) which carried the effluent onto the antenna (Moorhouse et al., 1969).

*Gas Chromatography—Mass Spectrometry (GC-MS).* High-resolution electron impact (EI) mass spectra were obtained on a Kratos MS-30 spectrometer interfaced via a Ryhage jet separator to a Pye 204 chromatograph. The data were analyzed using a NOVA-3 computer (Data General, Southboro, Massachusetts) equipped with a DS-55 data system (Kratos, Manchester, U.K.). Helium was used as the carrier gas (30 $cm^3$/min).

*Microchemical Reactions.* Saponification was carried out by reaction of 10 $\mu$l of the abdominal tip extract and 5 $\mu$l of a saturated methanolic KOH solution at room temperature for 15 min. The reaction was monitored by GC (column C, 180°C) and the reaction products identified by GC-MS. The saponified product was recovered by preparative GC and reacetylated by the addition of acetyl chloride (5 $\mu$l). Subsequent GC analysis showed the acetylated product to have the same retention time as that of the natural product.

Microepoxidation was achieved by the addition of 50 $\mu$g of metachloroperbenzoic acid (MCPBA) to 50 $\mu$l of the extract containing ca. 450 ng of the major component. The reaction mixture was left overnight and the reaction products were analyzed by GC (column D, 100°C, then 8°C/min to 240°C) and GC-MS.

Methoxymercuration–demercuration of the natural product was carried out as follows: ca. 100 $\mu$l of the extract, containing approximately 500 ng of the major component, was concentrated in a stream of nitrogen to remove the solvent, and the residue was redissolved in methanol (1 ml); excess mercuric acetate (50 mg) was added, and the mixture was shaken overnight in the dark (screw-capped glass vial covered with aluminum foil). The reaction was completed by the addition of excess sodium borohydride (50 mg) to the cooled (0°C) methanolic solution followed by acetic acid (100 $\mu$l) to destroy the excess borohydride. The reaction mixture was then partitioned between water (0.5 $cm^3$) and ether (2 $\times$ 0.5 $cm^3$). The ether extract was removed and concentrated in a stream of nitrogen to approximately 20 $\mu$l and subjected to analysis by GC (column C, 200°C) and GC-MS.

*Synthesis.* Infrared spectra (IR) were measured on a Unicam SP200 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Perkin-Elmer R12 spectrometer (60 MHz). Mass spectra (MS) were recorded on a Kratos MS30 fitted with a Kratos DS55 data system.

(*E*)-3-Tridecenyl acetate and (*Z*)-3-tridecenyl acetate were synthesized by standard procedures via a common intermediate (I) Scheme 1).

(Z)-*3-Tridecenyl acetate (III).* The acetate was obtained as a colorless liquid, bp 76–78°C (0.1 mm). The product was >99% isomerically pure by GC analysis (column E, conditioned at 180°C for 1 hr, then cooled to 138°C, and used isothermally at this temperature). IR ($cm^{-1}$): 2870-2920(s, C—), 1740 (s, acetate C=O). NMR: $\delta(CDCl_3)$ 1.3 (18H, broad s, $CH_3$ and chain $CH_2$'s), 1.9 (3H, s, acetate $CH_3$), 2.1 (2H, m, $CH_2$ allylic at $\Delta5$), 2.3 (2H, m, $CH_2$ allylic at $\Delta2$), 4.1 (2H, t, $J$ = 6 Hz, $CH_2$—O—CO), 5.45 (2H, m, olefinic H's). EI—MS: *m/z* (*r/a*) $M^+$240.210 (0.02) = $C_{15}H_{28}O_2$, ($M^+$ − 60) 180 (15), 96 (4), 82 (52), 81 (45), 68 (70), 67 (48), 55 (35), 54 (63), 43 (100). CI—MS: ($NH_3$) *m/z* (*r/a*) ($M^+$ + 18) = 258.2107 (100).

(E)-*3-Tridecenyl Acetate (V).* The acetate was obtained as a colorless liquid, bp 75–77°C (0.1 mm). GC (column C, 180°C) indicated one product more than 95% pure. The product was >98% isomerically pure by GC analysis (col-

SCHEME 1. Reagents: 1. $LiNH_2$/liq $NH_3$, $Fe(NO_3)_3$, THF; 2. methanol–paratoluene sulfonic acid; 3. Lindlar catalyst, $H_2$, quinoline, pentane; 4. acetic anhydride, pyridine; 5. Na, liq $NH_3$.

umn E, conditioned at 180°C for 1 hr, then cooled to 138°C and used isothermally at this temperature). IR ($cm^{-1}$): 2870–2920 (c, C—H), 1740 (s, acetate C=O). NMR: $\delta$ ($CDCl_3$) 1.3 (18H, broad s, $CH_3$ and chain $CH_2$'s), 1.8 (3H, s, acetate $CH_3$), 2.1 (2H, m, $CH_2$ allylic at $\Delta5$), 2.3 (2H, m, $CH_2$ allylic at $\Delta2$), 4.1 (2H, t, $J$ = 6 Hz, $CH_2$—O—CO), 5.4 (2H, m, olefinic H's). EI—MS: *m/z* (*r/a*) $M^+$240.240 (0.01) = $C_{15}H_{28}O_2$, ($M^+$ − 60) 180 (12), 96 (40), 82(60), 81 (40), 68 (70), 67 (50), 54 (65), 43 (100). CI—MS: ($NH_3$) *m/z* (*r/a*) ($M^+$ + 18) 258.2507 (100).

*Field Tests.* Field testing of the synthetic compounds was carried out in India in 1981–1982. Both polyethylene vials and red rubber septa dispensers were used loaded with various amounts of (*E*)- and (*Z*)-3-tridecenyl acetates (1–4 mg), singly and in combination. The traps used were delta traps (sticky area = 20 × 18 cm) opened out and held horizontally with the sticky surface facing upwards. These were arranged in a randomized array, 20 m apart, with three replicates of each treatment.

## RESULTS

*Structure Determination.* Analysis of the concentrated abdominal tip extract by capillary GC—MS (column F, 80°C then 6°C/min to 200°C) revealed one major peak. Mass chromatography of ions at *m/e* 180, 152, 109, 96, 82, 61, 55, 54, and 43 suggested that they were derived from one compound. The intense *m/z* 43 fragment ion ($CH_3CO^+$) (base peak) and the fragmentation pattern of the compound indicated an aliphatic acetate. The pseudomolecular ion *m/z* 180 ($M^+$ − 60) indicated a monoene 13-carbon acetate.

Comparative GC retention behavior studies of the natural compound and some standard straight-chain acetates, on a number of GC phases, also indicated the presence of a 13-carbon acetate (Table 1) and this was confirmed by coelution studies.

Alkaline hydrolysis of the extract produced a compound which had a lower retention temperature on a nonpolar column (column C) than the natural compound, and reacetylation of the hydrolyzed product gave a compound which had the same retention behavior as the natural compound, confirming the acetate functionality.

The double-bond position of the compound was located by two microchemical methods: microepoxidation (Bierl-Leonhardt et al., 1980), and methoxymercuration–demercuration (Blomquist et al., 1980, Vostrowsky et al., 1981, Baker et al., 1982), followed by analysis of the products by mass spectrometry. GC—MS of the product of the microepoxidation reaction (column D, 100°C then 6°C/min to 240°C) revealed diagonistic fragment ions, *m/z* 129 (60%), *m/z* 169 (10%), produced by cleavage $\alpha$ to the epoxide group of the epoxy acetate, and indicative of a 3,4-epoxide, implying that the position of unsaturation

TABLE 1. GC DATA FOR PHEROMONE COMPONENT AND STANDARDS

| Compound | Column[a] A (min) | B (°C) | G (°C) |
|---|---|---|---|
| Z7-12:Ac | 18.6 | | 141 |
| Z11-13:Ac | 21.0 | | |
| Z7-14:Ac | 22.4 | | 155 |
| Z7-16:Ac | 28.1 | | 168 |
| *Natural product* | 20.2 | 180 | 149 |
| E3-13:Ac | 20.3 | 180 | 149 |
| Z3-13:Ac | 20.3 | 180 | 149 |

[a]Temperature programs: A, 100°, 6°C/min to 200; B, 80°, 6°C/min to 200; G, 105°, 6°C/min to 200.

in the natural compound is Δ3. Other important fragment ions produced were *m/z* 196 and *m/z* 213, produced by loss of acetic acid and $CH_3CO$, respectively.

GC—MS analysis of the methoxylated products (column C, 200°C) formed from the methoxymercuration–demercuration reactions indicated the formation, in the spectrometer, of four characteristic fragment ions from cleavage α to the methoxy group; *m/z* 131 (5%), *m/z* 185 (4%), *m/z* 145 (8%), *m/z* 171 (20%). These fragment ions (two pairs separated by 14 mass units) indicated that carbon–carbon cleavage had occurred on either side of the methoxy group at positions 3 or 4, with the charge being retained on the oxygen-containing fragment and, therefore, that the natural product was unsaturated at the Δ3 position.

It is worth noting that the reltive abundance of the *m/z* 61 fragment and the ratios of the relative abundances of *m/z* 55 to *m/z* 54 fragments of the natural product were similar to those of (*E*)- and (Z)-3-tridecenyl acetate (Table 2), but not those of (Z)-11-tridecenyl acetate or (Z)-2-tridecenyl acetate (Horiike and Hirano, 1982).

TABLE 2.

| Compound | Relative abundance ratio: mass fragments *m/z* 55/*m/z* 54 | Relative abundance: % mass fragment *m/z* 61 |
|---|---|---|
| Natural product | 0.56 | 2 |
| Z2-13:Ac | 0.97 | 4 |
| Z3-13:Ac | 0.54 | 2 |
| E3-13:Ac | 0.50 | 2 |
| Z11-13:Ac | 1.26 | 11 |

TABLE 3.

| Compound | Retention time (min) | |
|---|---|---|
| | Column E[a] | Column D[b] |
| Z3-13:Ac | 3.45 | 7.7 |
| E3-13:Ac | 4.50 | 7.4 |
| Natural product | 4.60 | 7.4 |

[a]Column temp. 138°.
[b]Column temp. 152°.

The configuration of the double bond was established by comparing the retention behavior of the natural product and (*E*)- and (*Z*)-3-tridecenyl acetate on two different GC stationary phases (Table 3).

*Electroantennography.* The female abdominal tip extract and a solution of synthetic (*E*)-3-tridecenyl acetate at a comparable concentraiton ( ~ 10 ng) were examined by GC—EAG (capillary CW 20 M SCOT column). A single strong EAG response was recorded in both cases coincidental to the retention times of the synthetic and natural compounds. Responses of 3.7 mV and 4.5 mV were obtained to the natural product and (*E*)-3-tridecenyl acetate, respectively. Background responses were very low ( < 0.4 mV).

Synthetic (*E*)- and (*Z*)-3-tridecenyl acetate (1 ng each) were assayed by EAG "puff" testing. Averaged EAG responses obtained were 2.9 mV and 2.0 mV, respectively.

*Synthesis.* Following the tentative identification of (*E*)3-13:Ac as the sex pheromone of *S. heliopa*, pure synthetic samples of both the *E* and *Z* isomers were required for confirmation of the structure of the natural compound and to test the biological activity by a field bioassay. The synthesis of both geometrical isomers proceeds via the common precursor, tridec-3-yn-1-ol tetrahydropyranyl ether (I) (Scheme 1).

*Field Tests.* The results of catches of male *S. heliopa* in traps baited with (*E*)- and (*Z*)-3-tridecenyl acetate (V), (III), on a single night, are shown in Table 4. They indicate that (*E*)-3-tridecenyl acetate (V) is a very potent sex attractant pheromone for males of *S. heliopa*. From these results it would appear that 2 mg of (*E*)-3-tridecenyl acetate is the optimum loading. The *Z* isomer does not attract males of *S. heliopa* but rather inhibits the action of the *E* isomer.

## DISCUSSION

The sex pheromones of several species of Gelechiids have been identified to date, including those of *Pectinophera gossypiella* (Bierl et al., 1974), *Sito-*

TABLE 4.

| Treatment[a] | Total number of males caught in traps |
|---|---|
| Compound A | |
| 1 mg | 7 |
| 2 mg | 13 |
| 4 mg | 8 |
| Compound B | |
| 1 mg | 328 |
| 2 mg | 372 |
| 4 mg | 219 |
| Compounds A & B | |
| 0.5 mg each | 86 |
| 1 mg each | 52 |
| 2 mg each | 49 |
| Control | |
| No pheromone | 33 |

[a]A, (*Z*)3–13 : Ac; B, (*E*)3–13 : Ac.

*troga cerealella* (Vick et al., 1974), *Brachmia macroscopa* (Hirano et al., 1976), *Anarsia lineatella* (Roelofs et al., 1975), *Phthorimaea operculella* (Persoons et al., 1976), and *Scrobipalpa ocellatella* (Renou et al., 1980). *Scrobipalpa ocellatella* (Boyd) products (*E*)-3-dodecenyl acetate (VI) as the main sex pheromone component which is highly attractive to males of the same species. Males of *Scrobipalpa atriplicella* are strongly attracted by (*Z*)-5-dodecenyl acetate (VII) (Roelofs and Comeau, 1970, 1971).

(*E*)-3-Tridecenyl acetate [(*E*) 3–13 : Ac, V] has been identified as the sex pheromone of the tobacco stem borer, *S. heliopa* (ca. 70 ng/female) and is the first reported identification of this compound as a lepidopteran sex pheromone.

Relatively few odd-number straight-chain pheromones have been reported for lepidoptera, although the sex pheromone of another Gelechiid, *Phthorimaea*

OAc (VI)

OAc (VII)

SCHEME 2.

*operculella*, consists of (*E*,*Z*)-4,7-tridecadienyl acetate and (*E*,*Z*,*Z*)-4,7,10-tridecatrienyl acetate (Persoons et al., 1976).

Only one component has been identified so far in the pheromone systems of both *S. heliopa* and *S. ocellatella*, and attempts to identify minor components have not been successful.

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# PHEROMONE RECEPTOR CELL SPECIFICITY IN INTERPOPULATIONAL HYBRIDS OF *Ips pini* (COLEOPTERA: SCOLYTIDAE)

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**Abstract**—Electrophysiological studies of pheromone receptor cells keyed to ispdienol were performed in laboratory-raised hybrids of the eastern and western populations of the pine engraver, *Ips pini*. As previously shown in the parental beetles, the receptor cells keyed to ipsdienol could be classified as two distinct types: one keyed to (+)- and one to (−)-ipsdienol. None of the 20 ipsdienol cells recorded from $F_1$ hybrids were of an intermediate type. Recordings of the summated receptor responses (EAGs) showed no significant difference between parental beetles and hybrids. Similar results were obtained in reciprocal crosses, eastern females with western males and the reverse. Thus, there was no indication that sex-linked alleles determined the specificity of the ipsdienol receptor cell. The ratio between (+) and (−) cells was 14:6 in the hybrids compared to 1:12 in the western and 9:12 in the eastern populations.

**Key Words**—Pine engraver beetle, *Ips pini*, Coleoptera, Scolytidae, (+)-ipsdienol, (−)-ipsdienol, pheromone receptor, response specificity, electrophysiology, interpopulational hybrid

## INTRODUCTION

Populations of the pine engraver, *Ips pini* (Say), from eastern and western North America differ in release of and responses to their male-produced aggregation pheromones (Lanier et al., 1972). This interpopulational variation in pheromone response has been shown to be due to the use of different enantio-



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meric compositions of the pheromone ipsdienol (2-methyl-6-methylene-2, 7-octadiene-4-ol) (Lanier et al., 1980, Birch et al., 1980). Western beetles produce and respond to (−)-ipsdienol while eastern beetles produce and respond to a mixture of 65% (+)- and 35% (−)-ipsdienol. The (+) enantiomer alone attracts some eastern males and females, but the (−) enantiomer alone is hardly attractive. When mixed in a ratio of about 1:1, the attraction becomes strikingly potentiated (Lanier et al., 1980). Western beetles are attracted by (−)-ipsdienol but inhibited by the (+)-enantiomer (Birch et al., 1980).

Interpopulational $F_1$ hybrids and back-crosses ($B_1$) between eastern and western *Ips pini* attracted wild eastern *Ips pini* in direct order of "blood" relationship; that is, in order of attractiveness, eastern > $B_1$ eastern > $F_1$ > $B_1$ western = western (Piston and Lanier, 1974). In laboratory bioassays, $F_1$ females responded most strongly to hybrid males (G.N. Lanier, unpublished data), as was the case with interspecific hybrids of spruce-feeding *Ips* (Lanier, 1970). These bioassays suggest that, like pheromone production, pheromone response follows genetic relatedness.

Previous electrophysiological studies of eastern and western females have shown that the beetles perceive ipsdienol enantiomers via receptor cells that are either keyed to (+)- or to (−)-ipsdienol and suggested that the western population possesses receptor cells predominantly keyed to (−)-ipsdienol, whereas eastern beetles possess roughly 50% of each type (Mustaparta et al., 1980).

Therefore, it was of interest to study electrophysiologically the receptor cells of the interpopulational hybrids in order to find out whether parental or intermediate (hybrid) types of ipsdienol receptor cells would be present. Since it was of interest to find out whether the number of various cell types differed significantly between beetles of parent populations and offspring, electroantennograms (EAGs) (Schneider, 1957) were also recorded.

## METHODS AND MATERIALS

*Animals.* Eastern *Ips pini* (NY) used in the study originated from red pine, *Pinus resinosa*, at Tully, New York, and western *I. pini* (ID or CA) from ponderosa pine, *P. ponderosa*, at Worley, Idaho, and at McCloud Flats, California. The following interpopulational crosses were used to produce $F_1$ hybrids: $F_0$: NY ♀♀ × ID ♂♂, NY ♀♀ × CA ♂♂, ID ♀♀ × NY ♂♂, and CA ♀♀ × NY ♂♂.

Parent colonies and hybrids were reared in red pine logs in Syracuse, New York. Callow virgin females were removed from brood chambers, placed with fresh pine phloem in small metal containers, and air-mailed to Trondheim, Norway, for electrophysiological work. All colonies were established with 200 or more individuals and maintained in the laboratory for 20 or more generations prior to our tests.

*Substances.* Ipsdienol (+) and (−) enantiomers were kindly provided by G. Ohloff, Firmenich Company, Geneva, Switzerland, and were synthesized according to Ohloff and Giersch (1977). Each contained roughly 6% of the opposite enantiomer. The cells were also tested for some ipsdienol analogs (racemates, chemical purity 98–99% free of ipsdienol), including amitinol (see below), which were previously used in a study of structure–activity relationship (Mustaparta et al., 1984).

*Preparation and Recording.* The animal preparation (Angst and Lanier, 1979) and recording of responses from single olfactory cells were described previously (Mustaparta et al., 1979, 1980). Recordings obtained were usually from single cells, but in some cases activity from two cells appeared simultaneously. This is in agreement with morphological studies that showed the presence of either one or two cells of about the same size within single olfactory sensilla in *Ips* species (Light, personal communication). Activities from two simultaneously recorded cells were easily distinguished when their spike amplitudes were markedly different. Often the recordings from (+)-ipsdienol cells were obscured by the activity of another cell type. Usually, the other cell responded strongly to amitinol, a pheromone component for other *Ips* species (Francke et al., 1980), but not known to be relevant in the *Ips pini* communication system. In these cases, selective adaptation (Vareschi, 1971, see also Kaissling, 1979) was employed. The key odorants of the two cells were applied alternately, without delay, in order to adapt one cell during the response of the other. Thus, using amitinol as adaptation stimulus, after which (+)-ipsdienol was applied, the amitinol cell was kept adapted and interfered minimally with the response recorded from the ipsdienol cell. Selective adaptation revealed the same specificity of the (+)-ipsdienol cells as was found when another cell did not interfere. Recordings of EAGs followed the methods of Angst and Lanier (1979).

*Stimulation.* The stimulation technique, the use of the syringe olfactometer (Kafka, 1970), and the stimulation procedure were previously described (Mustaparta et al., 1980). The cells were tested with four or five concentrations of each compound. The enantiomers of ipsdienol and the analogs were used in duplicate series.

## RESULTS

Olfactory receptor cells were classified according to their relative sensitivity to various host volatiles and bark beetle-produced compounds (Mustaparta et al., 1979). Cells responding to ipsdienol at low concentrations (0.1 and 1 $\mu$g) responded minimally, if at all, to other compounds. These cells were intensively studied by exposing them to duplicate series of ipsdienol enantiomers, racemic mixtures, and some of the analogs used by Mustaparta et al. (1984).

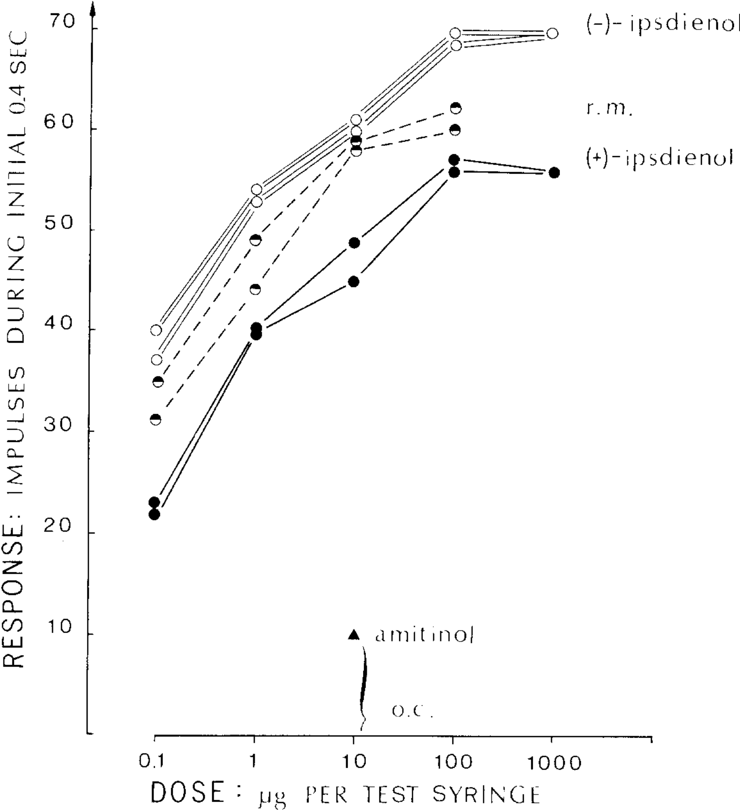


FIG. 1 Dose–response curves for one (−)-ipsdienol-specialized cell of a daughter from the cross NY ♀ × CA ♂. Equivalent response to (+)-ipsdienol that occurred at a 10-fold higher concentration is presumed to be the result of roughly 6% contamination of the (−)-enantiomer. Response to the racemic mixture (r.m., 50% of each enantiomer) is intermediate. o.c.: other compounds tested.

The dose–response relationships for the ipsdienol cells of $F_1$ hybrids showed that they could be classified as responsive either to (+)- or (−)-ipsdienol (Figures 1 and 2), as had previously been the case for the parent populations (Mustaparta et al., 1980). No ipsdienol cell responded equally to (+)- and (−)-ipsdienol or showed dose–response relationships which differed significantly from those described previously and also found here in parental beetles.

Altogether, 20 ipsdienol cells were examined in $F_1$ hybrids and 15 in parental beetles; the latter were replications of previous recordings (Mustaparta et al., 1980). The ratios of (+)- and (−)-ipsdienol cells differed significantly between eastern and western beetles (Table 1). In $F_1$ hybrids 14 (+)- and 6 (−)-ipsdienol cells were recorded of which 11 [eight (+)- and three (−)-ipsdienol

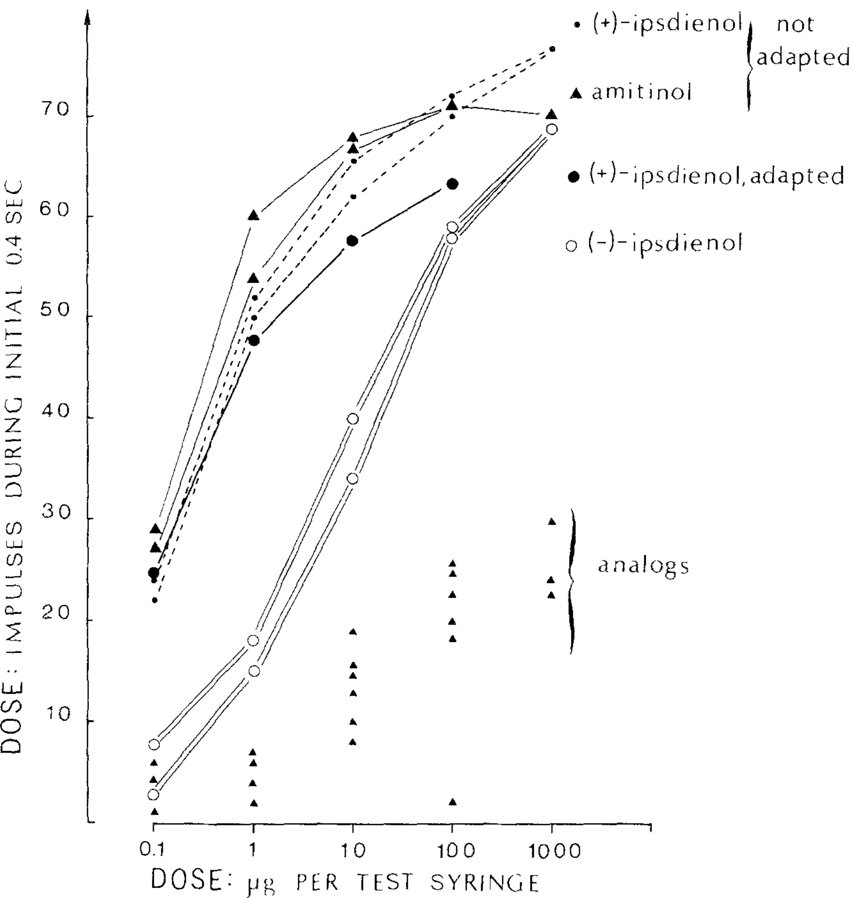


FIG. 2 Dose-response curves for two simultaneously recorded cells from a daughter of the cross NY ♂ × ID ♀. One cell is specialized to (+)-ipsdienol and the other to amitinol. Potency of (+)-ipsdienol was only slightly reduced after adaptation to amitinol, i.e., the amitinol cell, being adapted to its key compound, interfered less with the (+)-ipsdienol cell during the following stimulation. Equivalent response of the cell to (−)-ipsdienol at 10–100 times the concentration of (+)-ipsdienol is presumed to be the result from contamination of the sample with about 6% (+)-ipsdienol. Analogs of ipsdienol were tested after adaptation to amitinol and are therefore considered to have evoked slight response from the cell keyed to ipsdienol.

cells] were from offspring of the crosses NY ♀♀ × ID/CA ♂♂. The other nine cells, six (+)- and three (−)-ipsdienol cells, were from beetles of the reverse crosses, ID/CA ♀♀ × NY ♂♂. The ratio of (+) and (−) cells in $F_1$ differed significantly from that of western, but not eastern, populations.

All recordings from cells keyed to (−)-ipsdienol were obtained without

TABLE 1. FREQUENCY OF (+)- AND (-)-IPSDIENOL RECEPTOR CELLS ENCOUNTERED IN EASTERN AND WESTERN POPULATIONS OF *Ips pini* AND THEIR $F_1$ HYBRIDS

| Population | Individuals | Cell type | |
|---|---|---|---|
| | | (+)-Ipsdienol | (–)-Ipsdienol |
| Eastern | | | |
| 1980[a] | | 3 | 6 |
| 1982–1983 | | 6 | 6 |
| Total | 18 | 9 (43%)[b] | 12 (57%) |
| Western | | | |
| 1980 | | 1 | 9 |
| 1982–1983 | | 0 | 3 |
| Total | 12 | 1 (7.7%) | 12 (92.3%) |
| Hybrids (all 1982–83) | | | |
| Eastern mother | 8 | 8 | 3 |
| Western mother | 7 | 6 | 3 |
| Total | 15 | 14 (70%) | 6 (30%) |

[a]1980 data from Mustaparta et al. (1980).
[b]Chi-square values for intergroup comparisons are as follows: Eastern vs. Western, $\chi^2 = 4.07$, $P < 0.200$; Eastern vs. hybrids, $\chi^2 = 3.11$, $P < 0.500$; Western vs. hybrids, $\chi^2 = 20.50$, $P < 0.001$.

interfering activity from other receptor cells. Their dose–response relationships were similar within the hybrid groups and the parental beetles. Figure 1 shows the typical dose–response relationships for a (−)-ipsdienol cell obtained from an $F_1$ offspring of NY ♀♀ × CA ♂♂. As in the case for (−)-ipsdienol cells in parental beetles, the curves for (−)- and (+)-ipsdienol form roughly parallel lines separated by one log unit. The intermediate effect of the racemic mixture and the low effects of other compounds and analogs are shown in Figure 1.

In many recordings of (+)-ipsdienol cells, activity of another cell type was obtained simultaneously. Usually the interfering cell was keyed to amitinol, both in parental and hybrid beetles. In some of these recordings, the spike amplitudes were markedly different, so responses could clearly be ascribed either to a (+)-ipsdienol cell or to an amitinol cell. It also happened that an amitinol-responsive cell died, after which a cell keyed to (+)-ipsdienol became evident. In addition, several (+)-ipsdienol cells and amitinol cells were recorded separately, allowing unencumbered evaluation of their responses. However, when spike amplitudes of (+)-ipsdienol and amitinol cells were difficult to distinguish, the dual recording was confirmed by the irregular firing rates and frequent overlapping (summation) of spikes. This was particularly pronounced when high intensities of amitinol elicited activity from both cells.

Further separation of the responses of amitinol and (+)-ipsdienol cells was evident when these compounds were alternately used as adaptation stimuli. Typically, the initial application of (+)-ipsdienol and amitinol (each at 10 $\mu$g) elicited 57 and 67 impulses /0.4 sec, respectively. When the test was repeated with a rapid application of 10 $\mu$g ipsdienol–ipsdienol–amitinol–amitinol (different syringes), the responses obtained were 57, 35, 67, 37 impulses/0.4 sec. This shows that the amitinol cell (in contrast to the ipsdienol cell) did not become adapted to ipsdienol. The application of these stimuli in the reverse order showed that neither did the ipsdienol cell become significantly adapted to amitinol. The specificities of the (+)-ipsdienol cells studied by selective adaptation were similar to those of (+)-ipsdienol cells studied without interfering cell activity.

Figure 2 shows dose–response relationships of a cell in an $F_1$ NY ♀♀ × ID ♂♂ keyed to (+)-ipsdienol. This cell was recorded simultaneously with an amitinol cell and selective adaptation was employed. The adaptation to amitinol strongly reduced the response to amitinol, but only slightly to (+)-ipsdienol [Figure 2, (+)-ipsdienol, adapted]. Low responses to ipsdienol analogs are indicated, which were also obtained during amitinol adaptation. Therefore, both the dose–response curves for (+)-ipsdienol (adapted) and of the analogs in Figure 2 are considered to be responses by the (+)-ipsdienol cell, exclusively.

Despite the differences observed among the populations and hybrids in frequencies of (+)- and (−)-ipsdienol cells, recording of summed receptor responses by EAGs revealed no significant differences in dose–response curves for the two enantiomers.

## DISCUSSION

Genetic drift due to inbreeding during 20+ generations of colonies that had been maintained in the laboratory is a minor concern because several thousand individuals were continuously maintained. Unless selection for olfactory receptors occurred in the environment of the colony cages, the allelic frequencies should have remained similar to those of the founding populations.

The dose–response relationships of (+)- and (−)-ipsdienol cells in eastern and western *I. pini* and their $F_1$ indicate that intermediate receptor cells that possess roughly 50% of each membrane receptor type do not appear in interpopulational hybrids. For all ipsdienol cells recorded, the approximately parallel dose–response curves for the enantiomers were shifted at least one log unit from each other. In addition, the response spectra of the ipsdienol cells to various ipsdienol analogues were identical in parents (34 cells from 30 individuals) and hybrids (20 cells from 15 individuals). We conclude that both (+)- and (−)-ipsdienol cells of parental and $F_1$ beetles possess the same membrane receptors. Thus, a hybrid receptor cell is probably not formed in interpopulational $F_1$ hybrids.

It was previously suggested (Mustaparta et al., 1980) that the effect of (−)-ipsdienol on receptor cells keyed to (+)-ipsdienol, and vice versa, was most likely due to the optical impurity of the two enantiomeric compounds used. Thus, it is probable that each type of ipsdienol cell possesses mainly one type of membrane receptor, either keyed to (+)- or to (−)-ipsdienol. In contrast to the present results, Priesner (1979) reported that crosses between the cembrane pine and the larch races of the larch bud moth, *Zeiphera diniana* Gn., produced offspring having three types of receptor cells: the two parental types and one intermediate type. The genetic system that determines the phenotypes of pheromone receptor cells in the larch bud moth seems to be simpler than that in the pine engraver beetle, of which eastern and western populations possess two types of receptor cells in different proportions. Absolute proof against the occurrence of intermediate ipsdienol receptors is not possible. Nevertheless, if they do occur, hybrid receptor cells should have been detected among the 20 ipsdienol receptor cells recorded from 15 individuals in interpopulational hybrids. Furthermore, similarity of results obtained for reciprocal crosses give no indications of sex-linked alleles for receptor cell specificity.

Previous EAG recordings from eastern and western *Ips pini* using the cartridge stimulation technique (Angst and Lanier, 1979) were similar to our EAG study using the syringe olfactometer. Both studies found no significant difference between sensitivities of whole antennae to ipsdienol enantiomers in eastern and western beetles. The present study also found no difference between parental and hybrid antennae, which indicates that total numbers of receptors were similar. There is no indication of malfunctioning "hybrid" receptor cells lacking abilities to form membrane receptors.

The results from EAG recordings in *Ips pini* appear contradictory to the findings from single-cell recordings that indicate the eastern and western beetles differ in their relative numbers of (+)- and (−)-ipsdienol cells. This apparent discrepancy may result from a combination of the relative crudeness of EAG recordings and the optical impurity of the ipsdienol enantiomers. Another explanation might be that the apparent differences in (+)- and (−)-ipsdienol cells are artifacts of sampling error. In order to obtain further information about genetically controlled differences in ipsdienol receptors, it would be necessary to use ipsdienol enantiomers of higher optical purity.

It seems that all *Ips pini* populations, both parent and hybrid, possess similar receptor mechanisms to obtain information from both (+)- and (−)-ipsdienol and to discriminate between the two enantiomers via two different cell types. These may be present in different proportions in the various populations. Nevertheless, the variations in behavioral responses between populations depending on enantiomer composition (Lanier et al., 1972, 1980) are most probably due to differences of the integration process in the central nervous system (CNS). The most extreme difference among these populations is the synergistic

effect of the two enantiomers in the eastern native populations and the antagonistic effect in the western beetles, which is the strongest indication of different integration of activity from the (+)- and the (−)-ipsdienol cells (Mustaparta et al., 1980). In hybrids, the CNS integration process might thus be intermediate to the two extremes, leading to less synergism or less antagonism of the enantiomers, respectively.

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# CHEMOATTRACTION BETWEEN FRY OF ARCTIC CHAR [*Salvelinus alpinus* (L.)] STUDIED IN A Y-MAZE FLUVIARIUM

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**Abstract**—Attraction of fry of Arctic char [*Salvelinus alpinus* (L.)] to water conditioned by conspecifics of the same age was studied in a Y-maze fluviarium. Two types of experiments were run. In "up-swimming tests" (1), starting from the common leg of the Y-maze, a single fish was given the choice of entering one of the two upstream arms. In "gradient tests" (2), the momentary positions of a single char were time-lapse photographed in a test yard of this common shank. Strong attraction to conditioned water was observed in both types of tests.

**Key Words**—Arctic char, chemoattraction, fish, fry, *Salvelinus alpinus*, Y-maze fluviarium.

## INTRODUCTION

Fluviarium tests were performed to study whether fry of Arctic char [*Salvelinus alpinus* (L.)] were attracted to water conditioned by conspecifics of the same age. First summer juveniles of landlocked Arctic char are attracted to water containing odor of conspecifics (Höglund and Åstrand, 1973), but no attraction was induced by three other species of subarctic fish native to Sweden (Höglund et al., 1975). Adult anadromous Arctic char are also attracted to a water current conditioned by conspecific smolt or by intestinal content and bile from the latter (Selset and Døving, 1980). All these observations suggest the occurrence of a pheromone, active as a stimulus for intraspecific attraction, both in juvenile and adult Arctic char.

There are very few studies concerning chemopreference to "natural" stimuli in salmonid fry. Even before hatching, the olfactory epithelium in salmonids



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is morphologically well developed with receptor cells (Watling and Hillemann, 1964; Brannon, 1972; Jahn, 1972; Evans et al., 1982). Although the epithelium has no differentiated lamella, olfactory receptors are also present in alevins of Arctic char (personal observations). This points to the capacity of olfactory-mediated attraction to conspecifics in early life within the species. The aim of the present study is, therefore, to find out further whether fry of Arctic char are also attracted to water conditioned by odorous agents from conspecifics of the same age. The reactions were studied quantitatively in two types of behavioral tests by using a Y-maze fluviarium.

## METHODS AND MATERIALS

*Experimental Fish.* The behavior was studied by hatchery-reared Arctic char, *Salvelinus alpinus* (L.), deriving from wild fishes in Lake Tinnsjø, Telemark, Norway (about 350 specimens arrived on May 26, 1981).

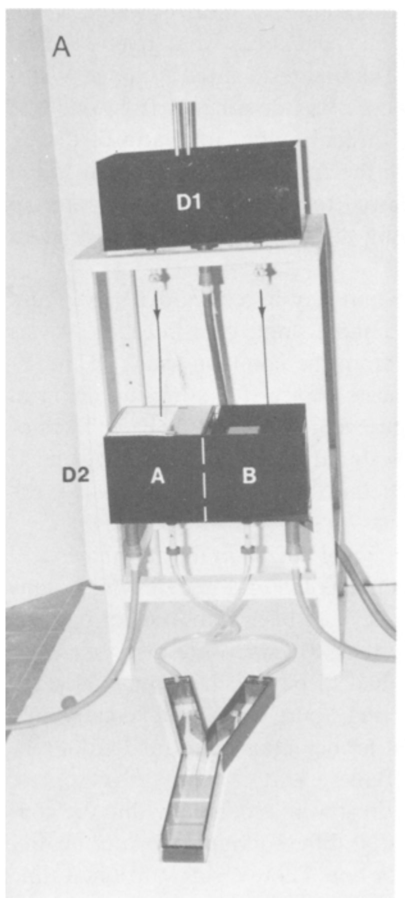
In the hatchery, the fishes were kept in troughs, supplied most of the time with cold water ($<2°C$). The average temperature was $<0.3°C$ from December to April and $<2°C$ during April. In May during daytime it was 5–10°C.

The fishes arrived in Uppsala approximately two months after hatching when they had been eating Ewos "char starter feed" for about a week. In our laboratory they were placed in two incubating boxes submerged in one trough (Ewos type 2002) with flowing aerated Uppsala tap water at a rate of 2.2 liters/min. There they were fed the same food twice a day to a total amount of 4–5% of body weight per day. The initial preference test started 14 days after arrival. The fresh weight ± SD on June 18, 1981, was 0.14 ± 0.03 g ($N$ = 20). Test 1 (see below) ended June 15, 1981. The fishes in test 2 were bigger in size. Char tested June 24–25, 1981, had a fresh weight (mean ± SD) of 0.28 ± 0.09 g ($N$ = 10).

*Water Quality.* All tests were performed in aerated Uppsala tap water. This is a subsoil water high in calcium (2.8 mM $Ca^{2+}$). For more detailed chemical characterization see Pärt and Svanberg (1981). The water temperature in the storing trough varied between 10.0 and 11.5°C, and in the Y-maze between 11.1 and 12.6°C. The oxygen concentration of water entering the Y-maze was 9.2–9.7 mg/liter ($N$ = 9).

*Y-Maze Fluviarium and Experiments.* Tests were performed in a Plexiglas Y-maze fluviarium (Figure 1A, and B) in dim light (0.32 $\mu W/cm^2$, 0.07 lux). The water flow through each inlet was 320 ml/min, corresponding to 0.4 cm/sec. The common down-channel was provided with a test yard confined by strainers of stainless steel (10 × 7 cm, T1 in Fige 1B) and the flow rate was 0.5 cm/sec. The square section (area of water) in the latter was 21 $cm^2$ as compared to 15 $cm^2$ for each arm; the water depth was 3 cm.

One hundred eighty odor-donating specimens were placed in compartment



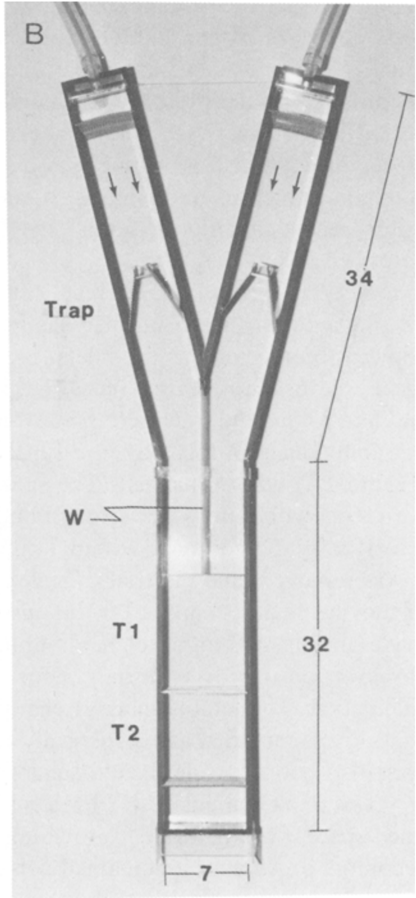




FIG. 1(A) The Y-maze fluviarium. Constant head box D1 distributes equal amounts of aerated tap water (420 ml/min) to each of two uniform compartments of D2, A and B, maintaining equal constant volumes and pressure heads. A batch of odor-donating fish was placed in A. (B) The Y-maze proper. Two types of test were performed. In test 1, "up-swimming tests," starting from test yard T1 one Fry at a time was given the choice of swimming up one or the other inlet arm. It was considered to have made its choice after having been trapped in one arm. The median transparent partition wall W just ahead of the test yard was removed in these tests. In test 2, "gradient tests," one specimen at a time was time-lapse photographed in the spatial differentiation of water quality within test yard T1. The momentary positions in pure water and water containing char odor were summed. Test yard T2 was not used in these present experiments. Measurements are given in centimeters.

A of the constant head box D2 (Figure 1A) containing 7.2 liters of water, which was supplied to A at a rate of 420 ml/min. The specimens were given food 60 min before being placed in compartment A. The first test started 1 h later. Water conditioned by the presence of the odor-donors was distributed from one test period to the other (see below) alternately through either inlet arm of the Y-maze. A single fish at a time was placed in the test yard, T1, situated in the common branch of the Y-maze. It was allowed to choose between "pure tap water" and "conditioned water" by following the two types of test procedure described below.

*Test 1:* "Up-swimming Test. After a 5-min stay in test yard T1 of Figure 1B, the anterior (upstream) net was removed and a single char in each test was allowed to enter one of the inlets. Starting from the common shank of the Y-maze, the fish was considered to have made its choice if it swam up one arm and had passed the trap there, shown in Figure 1B. After testing each batch of five individuals in this way, the supply of water from compartments A and B (Figure 1A) was exchanged. The same procedure was followed in control experiments with pure water through both inlets.

If a fry did not move within 3 min from being placed in the starting space, it was removed, and the trial canceled. Specimens that did not show any signs of moving within 5 min after the upstream net had been removed, or had not made any up-swimming choice within a further 10 min, were also canceled. However, on that account only a few tests had to be rejected from statistical calculation. The total number of char preferring "pure water" or "conditioned water" was recorded and statistically treated for deviation from random (a random distribution) using the chi-square test (Bailey, 1981).

*Test 2:* "Gradient Test." Preferred positions were recorded within the confined space T1 (Figure 1B), providing a spatial differentiation of water quality according to whether it contained fish odor or not. There a single fish at a time met pure water or conditioned water, approximately covering each lateral half. The positions taken by a fish were recorded by time-lapse flash photography every 30 sec using a 16-mm Bolex Paillard film camera. To reduce visual disturbance, flashes passed Kodak Wratten filter 89B (0.0% transmittance at 670 nm and 0.1% at 680 nm, cf. Kodak Filters publ. 1978). Otherwise the experiments were run in dim light.

A first and second test period was run, each of 35 min, and the water qualities (with or without fish odor) were switched from one side to the other. To secure new inverted spatial differentiation of water quality, the initial 10 registrations (representing the first 5 min of each 35-min period) were left out. A reaction value ($Rv$) was calculated for each experiment based on the two 30-min periods from the following expression:

$$Rv = (N_s - N/N_s + N) \times 100,$$

where $N_s$ and $N$ are the numbers of observations on either side of the test yard with or without char water.

Based on all *R*vs arrived at in repeated tests, the mean reaction value and standard deviation (M*Rv* ± SD) was calculated and the M*R*vs obtained in control experiments (no donors) and in tests with odor donors were compared with Student's *t* test. *R*vs obtained in the two test series were also compared with Wilcoxon's rank sum test (Bailey, 1981). Student's *t* test was also used to compare M*R*vs arrived at during each type of testing with the theoretical M*Rv* for an indifferent reaction (M*Rv* = 0).

## RESULTS

Preference reactions obtained are given in Tables 1 and 2. In control experiments (without donors), indifferent reactions were attained, i.e., neither attraction nor avoidance behavior was induced in test system 1 or 2. In "up-swimming tests" (1), the distribution of preferential choices between the two inlets did not deviate significantly from an even destribution. In "gradient tests" (2), control experiments did not show any significant deviations from nil (M*Rv* = 0), i.e., the reactions during control conditions were indifferent.

Significant attractions to water conditioned by conspecifics were attained both in "up-swimming tests" ($P < 0.001$, Table 1) and in "gradient tests" ($P < 0.001$, Table 2). The M*Rv* arrived at in experiments with donor fish was also significantly higher than in the control experiments both in Student's *t* test and Wilcoxon's rank sum test ($P < 0.01$, Table 2). One fish out of nine did not show any attraction to conditioned water. The *Rv* (−1.7) of the specimen was within the range of *R*vs for control experiments (range: −8.3 to +16.7) but

TABLE 1. CHEMOATTRACTION AMONG FRY OF ARCTIC CHAR: RESULTS OF TEST 1 ("UP-SWIMMING TESTS")

| No. of odor donating fish | No. of choices (%) for | | *P* value | Reaction[a] |
|---|---|---|---|---|
| | Conditioned tap water | Pure tap water | | |
| None (control) | 14 (56) | 11 (44) | >0.05 | Indifferent |
| 180 | 22 (88) | 3 (12) | <0.001 | Attraction |
| 180 | 23 (92) | 2 (8) | <0.001 | Attraction |
| 180 | 24 (96) | 1 (4) | <0.001 | Attraction |

[a]The distribution of observations was tested for deviation from random using chi-square analysis. Attraction means up-swimming into the inlet supplying water conditioned by conspecifics.

TABLE 2. CHEMOATTRACTION BETWEEN FRY OF ARCTIC CHAR: RESULTS OF TEST 2 ("GRADIENT TESTS")

| No. of odor donating fish | No. of experiments | $MRv \pm SD$[a] | P value comparison with | |
|---|---|---|---|---|
| | | | $MRv = 0$[b] | Reaction in control experiments[c] |
| None (control) | 9 | +5.4 ± 8.8 | >0.05 | – |
| 180 | 9 | +36.6 ± 21.6 | <0.001 | <0.01<br><0.01 |

[a]A reaction value ($Rv$) based on 120 photoregistrations was calculated as the percent difference of recorded stays in either water quality. A mean reaction value ($MRv \pm SD$) was calculated from all repeated tests with equal experimental conditions.
[b]$MRv$s are compared with the theoretical $MRv$ for an indifferent reaction (Student's $t$ test).
[c]The $P$ values are attained with Student's $t$ test and Wilcoxon's rank sum test.

outside the range of the remaining $Rv$s attained in tests with donor fish (range: +21.4 to +63.3).

The results of both test systems show that there are powerful chemoattractions between fry of Tinnsjø char.

## DISCUSSION

*Chemoattraction Observed.* The present study shows that chemoattraction exists between fry of Arctic char [*Salvelinus alpinus*(L.)]. It thus confirms earlier findings attained with older juveniles of the same species. Höglund and Åstrand (1973) found, by using the fluviarium technique (Höglund, 1961), clear olfactory-mediated attraction in first summer char (8.6–15.7 g, 10.6–12.5 cm) from one population in Lake Hornavan, Lappland, Sweden. Electrophysiological evidence presented by Døving and collaborators in Oslo, Norway, supports the assumption that chemoattraction behavior in juvenile Arctic char is mediated by the olfactory sense, as water conditioned by Arctic char elicited response in the olfactory bulb of conspecifics. Their results also indicated that different char populations may emit different odors (Døving et al., 1974).

*"Pheromones" in Homing.* Extensive studies performed by Hasler and his colleagues (reviewed by Hasler et al., 1978) artificially imprinted salmonid smolts to synthetic organic substances such as morpholine. During their spawning run, the mature fish then choose a stream containing this substance. The chemical nature of "real" home stream odor(s) guiding mature fish back to their natal river is not known, however. Chemical attractants released by alevins and juveniles may, alone or in combination with other odorous agents, be of signif-

icance for homing and the selection of spawning ground (Hasler, 1966; Nordeng, 1971, 1977; Solomon, 1973; Selset and Døving, 1980; Horrall, 1981). This may be true even though no conspecifics were present in the simulated home stream imprinting experiments performed by Hasler et al. (1978).

Char alevins must then release stimulants during a brief period of time as they leave their spawning ground or river shortly after emerging from the gravel (Frost, 1965; Johnson, 1980). The substances must adhere to the gravel and remain attractive for a long time. Such agents, acting as pheromones, have been suggested to be bile acid derivatives (Selset, 1980; Selset and Døving, 1980). They give well-manifested electrophysiological olfactory stimulation in Arctic char and grayling, *Thymallus thymallus* L., (Døving et al., 1980). The high sensitivity of the olfactory sense for bile acids, together with the results of Algranati and Perlmutter (1981) who identified "sexual attractant(s)" in the zebrafish (*Brachydanio rerio*) as cholesterol ester, suggest steroids as an interesting group of substances which may be of significance for the induction of chemoattraction in Arctic char and other species.

*Olfaction in Social Interactions*. Pitcher (1979) suggests that in "shoals" ("nonpolarized groups") are positions of members of the group percepted by the lateral line, vision and olfaction, but probably only the lateral line and vision are of importance for maintaining "schooling" ("polarized synchronized groups"). Although chemical attractants seem to interfere in social interactions during schooling or shoaling (Wrede, 1932; Keenleyside, 1955; Hemmings, 1966; Höglund and Åstrand, 1973), the biological implications of olfactory-mediated attraction in young Arctic char are not known. Arctic char keep together in schools or shoals, at least when they are pelagic (Aass, 1970; Klemetsen and Grotnes, 1980). The role of the olfactory sense in fish schooling and shoaling behavior remains to be further investigated.

*Implication of Water Quality*. The present experiments with Arctic char were run in tap water rich in calcium (approximately 3mM), much higher than in lakes normally inhabited by this species (Dickson et al., 1975; Fürst et al., 1981). As calcium ions are required for a normal olfactory receptor function and have modulatory effects on the olfactory response (Bodznick, 1978; Suzuki, 1978; Tucker, 1983), it would be of interest to complete the present chemoattraction study in soft water.

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# MODES OF DEFENSE IN NEMATINE SAWFLY LARVAE
## Efficiency Against Ants and Birds

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**Abstract**—Ventral glands are common in nematine larvae (Hymenoptera: Symphyta), but they show various degrees of development and are functional for defense only in some species. In those species, volatile irritants are produced which are effective against ants. Alternative or complementary mechanisms against ants are the pubescence of *Trichiocampus* spp., the foam pillars constructed by *Stauronema compressicornis*, various movements of the abdomen, which occur independently of the glandular secretion in several species, immobility of the flat larvae of *Nematinus luteus*, and burrowing within plant tissues in gallicolous larvae or miners. Glandular development is not clearly related to the appearance of the larvae, either cryptic or aposematic. The secretion, even when it is produced in large amounts by species with well-developed glands, is only moderately efficient against great tits. Bright colors are found in gregarious larvae; these were accepted only with reluctance by great tits and sometimes rejected, even species in which the ventral glands are reduced. We suggest that the various volatile irritants secreted by ventral glands are aimed primarily against insects (e.g., ants) and only secondarily against birds.

**Key Words**—Sawfly larvae, Nematinae, Hymenoptera, Tenthredinidae, defensive secretion, ventral glands,mechanical defense, crypsis, aposematism, gregariousness, predation, ants, birds.

## INTRODUCTION

The soft bodied sawfly caterpillars are potentially easy prey for predators and parastoids. Indeed, Benson (1950) gives an impressive list of their potential



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enemies, among them birds, ants, and parastoids. It is thus not surprising that diverse and elaborate defensive mechanisms are reported in this group (Benson, 1950); however, the evidence for these defenses is often anecdotal and detailed studies are few.

Chemical defense has been thoroughly studied only in *Neodiprion sertifer* (Diprionidae) (Eisner et al., 1974), and in *Perga affinis* (Pergidae) (Morrow et al., 1976). Both species sequester host plant terpenes in, respectively, two and one pouches of the foregut, and they regurgitate these compounds when the larvae are disturbed. Recently, a toxic octapeptide was found in the Australian larva *Lophyrotoma interrupta* (Pergidae) (Williams et al., 1982). Herbivorous vertebrates are frequently poisoned by ingestion of these larvae.

Nematine sawflies (Tenthredinidae) possess medioventral glands which can be everted when the larvae are alarmed. They release a fluid which, in some cases, is odorous (Yussa, 1922; Benson, 1950; Maxwell,1955; Alsop, 1970; Smith, 1970). The composition of the secretion has been studied for nine species in the genera *Nematus, Nematinus, Croesus, Pontania,* and *Pristiphora.* The secretions contain one or more volatile compounds, such as benzaldehydes, monoterpenes, aliphatic aldehydes, or acetates (Boevé et al., 1984; Bergström et al., 1984). These or similar compounds are commonly found in defensive secretions of insects and were classified by Eisner (1970) as nonspecific toxicants acting as irritants. Unidentified volatile compounds were also detected in *Platycampus luridiventris* and in additional *Nematus, Nematinus* and *Pristiphora* species (unpublished).

In this paper we study the interspecific variation in gland development and attempt to correlate it with larval appearance and with the efficiency of defense against two sorts of important predators: ants and birds. For convenience, the term aposematism will be used throughout the text, although this probable function of the bright colors will be documented later in the paper.

## METHODS AND MATERIALS

Larvae of 28 species were studied. They were all collected in the field in Belgium and maintained in the laboratory on their natural host plant. Both sawfly and host-plant species are listed in Table 1. Lorenz and Kraus (1957) and Nigitz (1974) were used to identify the larvae, and Benson (1951, 1952, 1958) for the adults.

Morphological studies were conducted on larvae fixed in Dubosq-Brasil and embedded in Paramat. The 7-$\mu$m sections were stained with ferric trioxyhematein–phloxin–light green. Gland size was evaluated by measuring the area of the gland, colored with hemalum and mounted in Canada balsam. For each species, one to four last-instar larvae were dissected. The surface measured is in point of fact the surface of the glandular tissue as observed, for example, in

TABLE 1. APPEARANCE AND FEEDING HABITS OF SPECIES STUDIED[a]

| | Appearance | Distribution | Host plants | Investigations |
|---|---|---|---|---|
| *Croesus septentrionalis* (L.) | A | G | *Alnus, Betula* | 1, 2, 3 |
| *C. varus* (Vill.) | C | G-S | *Alnus* | 1, 2, 3 |
| *Hemichroa australis* (Lepel.) | C | S | *Alnus, Betula* | 1, 2, 3 |
| *H. crocea* (Geoffr.) | A | G | *Alnus, Betula* | 1, 2, 3 |
| *Mesoneura opaca* (Kl.) | C | S | *Quercus* | 1 |
| *Nematinus luteus* (Pz.) | C | S | *Alnus* | 1, 2, 3 |
| *Nematus bipartitus* (Lepel.) | C | S | *Populus* | 1, 2, 3 |
| *N. hypoxanthus* (Först.) | C | S | *Populus, Salix* | 1 |
| *N. melanaspis* (Hg.) | A | G | *Populus, Salix* | 1, 2, 3 |
| *N. melanocephala* (Hg.) | A | G | *Salix* | 1, 2, 3 |
| *N. miliaris* (Pz.) | A | G | *Salix* | 1, 2, 3 |
| *N. pavidus* (Lepel.) | A | G | *Salix* | 1, 3 |
| *N. salicis* (L.) | A | G | *Salix* | 1, 2 |
| *N. spiraeae* (Zadd.) | C | Ag | *Aruncus* | 1, 3 |
| *N. tibialis* Newm. | C | S | *Robinia* | 1, 2, 3 |
| *Pachynematus scutellatus* (Hg.) | C | S | *Picea* | 1, 3 |
| *Platycampus luridiventris* (Fall.) | C | S | *Alnus* | 1 |
| *Pontania viminalis* (L.) | ? | ? | *Salix* | 1, 3 |
| *Pristiphora aquilegiae* (Voll.) | C | Ag | *Aquilegia* | 1 |
| *P. compressa* (Hg.) | C | S | *Picea* | 1, 3 |
| *P. conjugata* (Dahlb.) | A | G | *Populus* | 1 |
| *P. erichsonii* (Hg.) | A | G | *Larix* | 1 |
| *P. pallipes* Lepel. | C | Ag | *Ribes* | 1, 2, 3 |
| *P. saxesenii* (Hg.) | C | S | *Picea* | 1, 3 |
| *P. testacea* (Jur) | A | G | *Betula* | 1, 2, 3 |
| *Stauronema compressicornis* (Fabr.) | C | S | *Populus* | 1, 2, 3 |
| *Trichiocampus ulmi* (L.) | C | S | *Ulmus* | 1, 2 |
| *T. viminalis* (L.) | A | G | *Populus* | 1, 2, 3 |

[a]A: aposematic, C: cryptic, G: gregarious, S: solitary, Ag: aggregated (see text), 1: glandular morphology, 2: tests with ants, 3: test with great tits.

Figures 1A–C, and which represents half the total glandular surface (see Figure 1D). This measure was used as a rough approximation of these glands' defensive investment. Measurement of the volume of secretion would have been impossible in those species with reduced glands, and for which no detectable amount of secretion may be collected.

Defensive efficiency against ants was observed by placing a larva with 20 *Myrmica rubra* workers in a square container (side: 10 cm). Two minutes were allowed for the ants to discover the larva. During the next 3 min, the number of ants attacking or surrounding the larva was counted every 20 sec on a video-recording of the experiments. This quantifies the interest of the ants for the larvae as prey and is considered to be inversely related to the larvae's defensive

ability. Depending on the availability of the sawfly species, one to six repetitions were made with separate larvae. For some species, additional observations were made with larvae on their host plant. A twig of the plant bearing a larva was then placed in the foraging area of a laboratory nest of *Myrmica rubra*.

To test the efficiency of defense against birds, larvae were offered to two caged great tits (*Parus major*, one male, one female) collected as adults in the field. In each session, a bird received in succession a mealworm, three full-grown sawfly larvae of the same species, and again a mealworm. Acceptance or rejection was recorded. The limited supply of biological material excludes a precise quantification of the different larvae's palatability, and these tests were only carried out to show up possible trends in the birds' reactions. No bird was ever tested with more than three different species per day, in order to avoid satiation. No bird ever refused a mealworm.

Statistical tests are described in Siegel (1956).

## RESULTS

*Crypsis and Aposematism.* Nematine caterpillars offer some of the best examples of both crypsis and aposematism. Two extremely cryptic species are illustrated in Figure 1 (A and B).

The larva of *Platycampus luridiventris* (Figure 1A) is green, flat, with lateral expansions, and is tightly appressed to the leaf surface. These larvae are solitary and located on the underside of the leaf, often along the major veins when not feeding. *Nematinus luteus* larvae (Figure 1B) are semicylindrical in shape. They are green with small white dots, mimicking the texture of the leaf. The lateral margins are light, counteracting the shadow effect. They are solitary and live on the upper surface of the leaves.

Brightly colored larvae are illustrated in Figure 1 (C and D). Larvae of *Hemichroa crocea* (Figure 1C) feed in groups along the leaf-edge. They are light brown with black longitudinal lines on each side. They usually curl the extremity of their abdomen ventrally. The larvae of *Croesus septentrionalis* are yellowish with black dots and a black head. They also feed in groups at the edges of the leaves. When disturbed, they raise their abdomens in a typical defensive posture (Figure 1D).

The appearance, cryptic or aposematic, and the feeding habits, gregarious or solitary, of the different species studied are given in Table 1. Gregarious habit is significantly correlated with bright coloration and crypsis with solitary habit ($\chi^2$, $P < 0.001$). Solitary species, if not extremely cryptic as described above, are at least the same color as the leaf. There is no association between type of appearance and taxonomic position; cryptic and aposematic larvae are often found within the same genus.

The larvae of a few solitary species can, however, appear aggregated as a

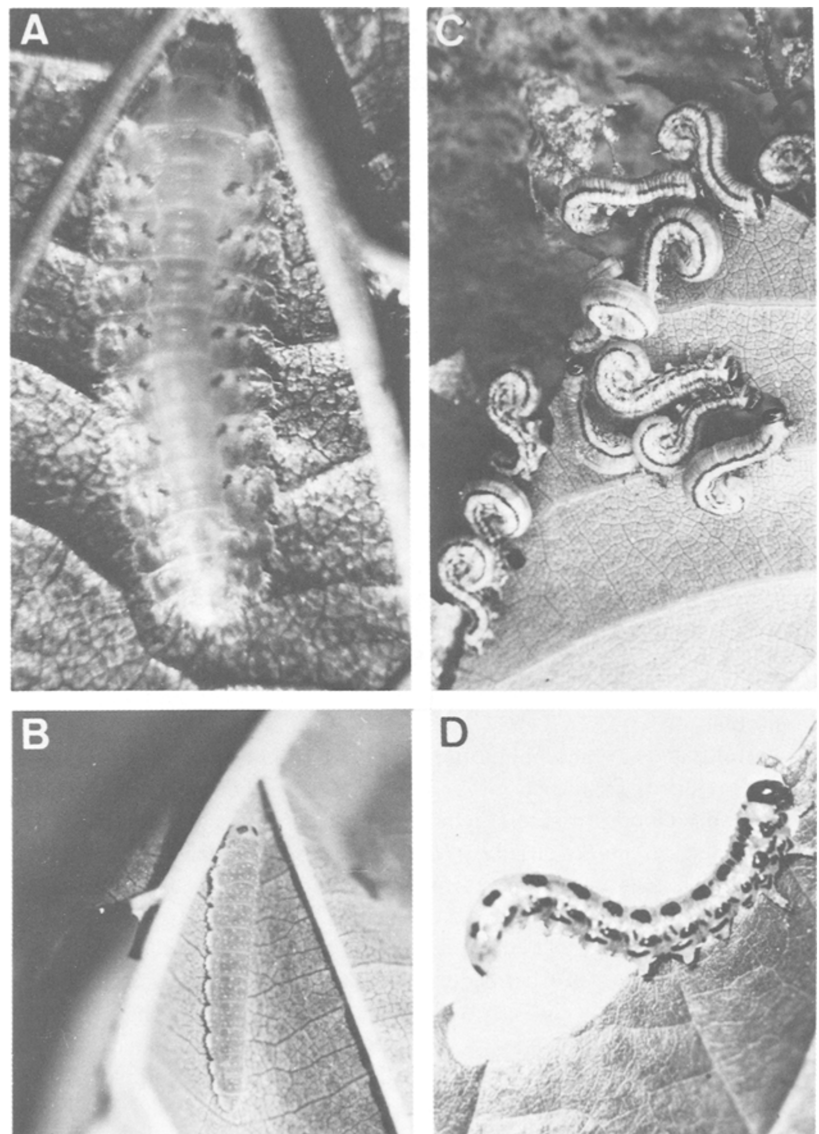


Fig. 1. Cryptic (A, B) and aposematic (C, D) larvae of nematines. (A) *Platycampus luridiventris*, (B) *Nematinus luteus*, (C) *Hemichroa crocea*, (D) *Croesus septentrionalis*.

result of the distribution of their host plant. Both *Nematus spiraeae* and *Pristiphora aquilegiae* were found on ornamental herbs, respectively *Aruncus silvester* and *Aquilegia vulgaris*, and a large number of larvae is often observed on a single plant. Nonetheless they feed mostly by themselves and are not brightly

colored. *N. spiraeae* and its cultivated host plant are introduced North American species. The cryptic larvae of *Pristiphora pallipes* can form dense populations on gooseberry shrubs in gardens. We do not know how these three species are distributed in their natural habitat.

The larvae of *Croesus varus* are found either singly or in small groups depending on their age. The green full-sized larvae are usually solitary, whereas the younger, also green, larvae are more gregarious. Very young larvae are grouped and blackish, thus contrasting with the substrate. This may be an intermediate between clearly cryptic and clearly aposematic species. The characteristics of this species are not fixed but change gradually during ontogeny.

The larvae of *Pontania* spp. live in galls and are thus concealed. Mature galls, however, are sometimes bright red, but the function of this coloration remains unknown.

*Ventral Glands*. Typically seven glands are present, located midventrally in the first seven abdominal segments. Each gland consists of a pocket in the integument, which at rest is invaginated into the body. The cuticular reservoir is lined by a single layer of glandular cells and is prolonged by a flat and broad duct leading to a slit-shaped opening (Figure 2D). These slits open posteriorly and on those segments with prolegs just behind them. Muscle fibers link both sides and the apex of the gland to the body wall. The general structure of the glands seems quite similar throughout the subfamily, but, as illustrated in Figure 2, considerable variation was encountered in the development of glandular tissue and musculature.

Ventral glands are undoubtly used for defense in those species where they are well developed. Disturbed larvae raise and wave their abdomens and evert their glands by blood pressure (Figure 3).Eversion of the glands was observed in *Croesus* spp., *Nematus pavidus, N. melanaspis, N. spiraeae,* and in *Pristiphora* spp., whereas eversion was never seen in some species with reduced glands, e.g., *Hemichroa* spp., and *Nematus salicis*. In those species, we were unable to detect any sign of secretion, and their glandular musculature is often reduced to a single pair of fibers (Figure 2B and C).

In Figure 4, the species are ranked according to the absolute size of the glandular tissue in full-grown larvae. In species with well developed glands, the first and last glands are smaller than the others. Interspecific comparisons were therefore made by measuring glands from the 2nd to 6th segments. Both cryptic and aposematic species are found among species with or without voluminous glands.

No significant difference between the distributions of gland sizes in aposematic and cryptic species can be demonstrated using the "median test" ($P < 0.23$). However, Fisher's exact probability test suggests that crypsis is often associated with smaller glands ($< 0.1$ mm$^2$) and aposematism with larger glands ($>0.1$ mm$^2$) ($P \simeq 0.046$).

The size of the glands is not simply proportional to the size of the body, as

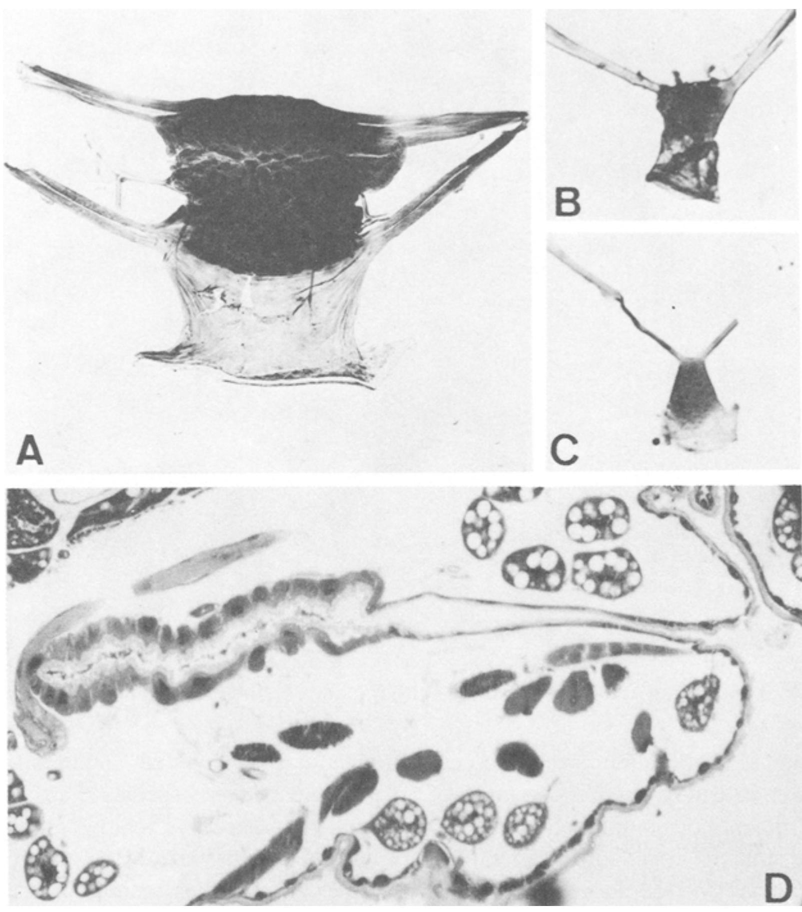


Fig. 2. Ventral glands of three nematine species, same scale; (A) *Nematus pavidus*, (B) *Nematus salicis*, (C) *Pontania viminalis*. (D) Sagittal section through a ventral gland of *Nematus pavidus*.

demonstrated by devising an index which reduces differences due to larval sizes. The index used was: the glandular surface (in $10^{-2}$ mm$^2$) divided by the square of the head capsule width (in mm$^2$). For preserved specimens, the width of the head capsule is a good measure of the body size: its cubic dimension is strongly correlated with the weight of living larvae ($r = 0.91$; 243 larvae of various ages belonging to 12 different species). The values of the index calculated for penultimate larvae of *Nematus pavidus* fall within the range of values calculated for last-instar larvae, which demonstrates that, at least for different instars of a species, the index eliminates variations due to age and size.

In Figure 5, the species are ranked according to relative glandular devel-

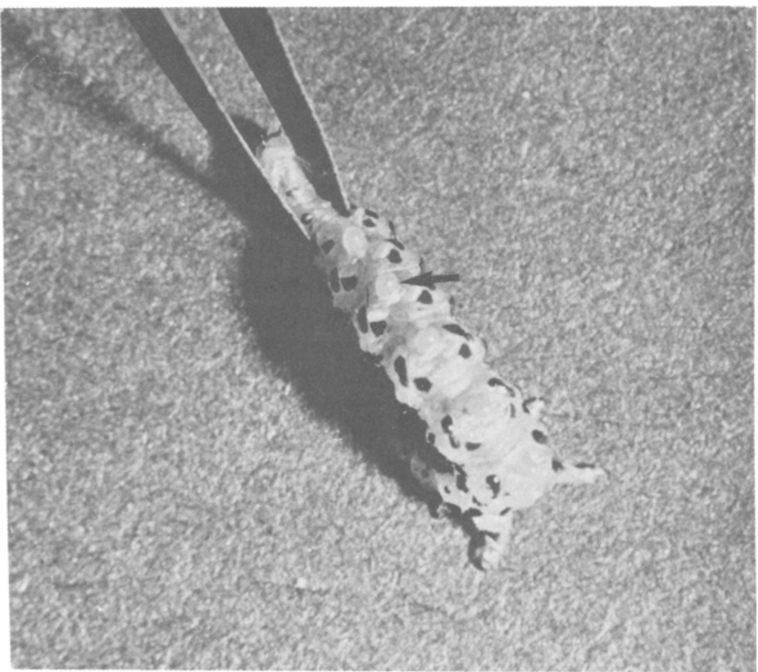

Fig. 3. Secretion at the tip of everted ventral glands (arrow) of *Nematus melanaspis*.

opment. There is no correlation between glandular development and taxonomic status, as can be seen by the ranks of the different *Nematus* species. Further, no significant correlation can be found between appearance and relative glandular size. Since a correlation was found between the absolute size of the gland and aposematism (see above), it is the ability to produce large amounts of volatile compounds, independently of body size, which could be functionally linked to bright colors.

*Defense against ants.* The number of ants surrounding the larva increased during the first 2 min after beginning the test and then stabilized. We estimate the efficiency of defense by calculating the mean number of ants surrounding the larvae after 2 min and at each subsequent count until the end of the experiment (see *Methods and Materials*). This number could in principle be related to the size of the larva. However, there was no correlation between the weight of the larva and the number of ants around it ($r = 0.01$; 54 larvae belonging to 17 different species).

In Figure 6, the species are ranked according to the mean number of ants surrounding them. Gland sizes are significantly smaller in the most heavily attacked species (mean number of ants > 3) than in the less attacked species (mean number of ants < 3) ($P < 0.005$, median test). Heavy attacks are as-

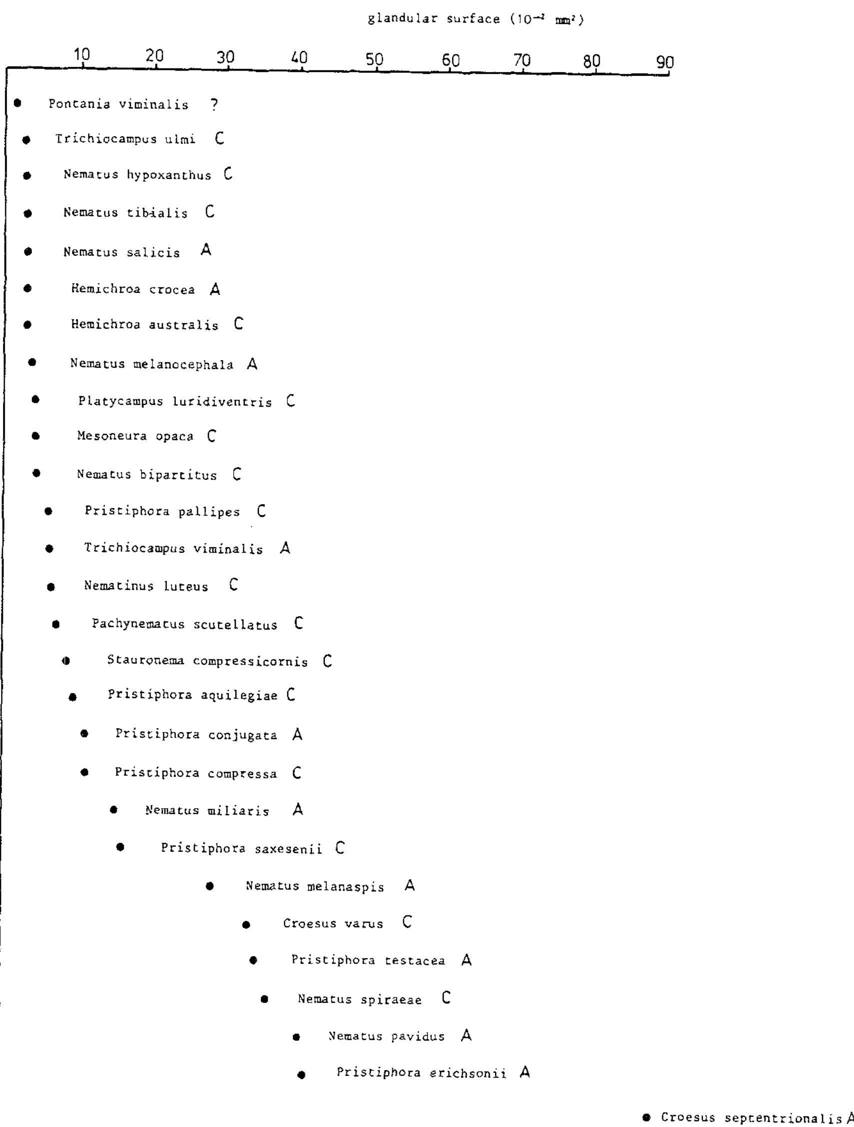


Fig. 4. Gland size and appearance in nematine larvae. (A) aposematic, (C) cryptic.

sociated with very small glands ($<0.05$ mm$^2$), whereas few attacks or no attacks are associated with voluminous or medium-sized glands ($>0.05$ mm$^2$) ($P < 0.001$, Fisher's exact probability test for two samples).

Some species with reduced glands are exceptions to this general rule and are apparently well protected against ants. Also there is no simple correlation

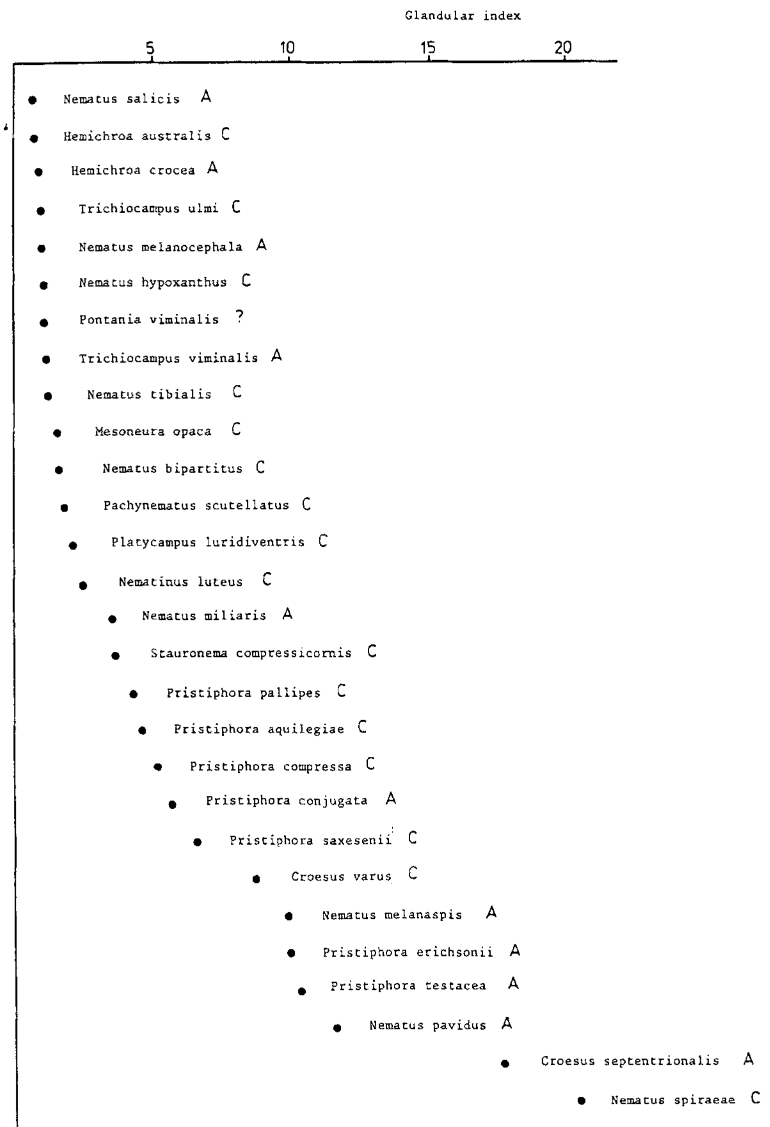


Fig. 5. Relative gland size and appearance in nematine larvae. Glandular index: glandular surface (in $10^{-2}$ mm$^2$) divided by the square of the head capsule width (in mm$^2$). (A) aposematic, (C) cryptic.

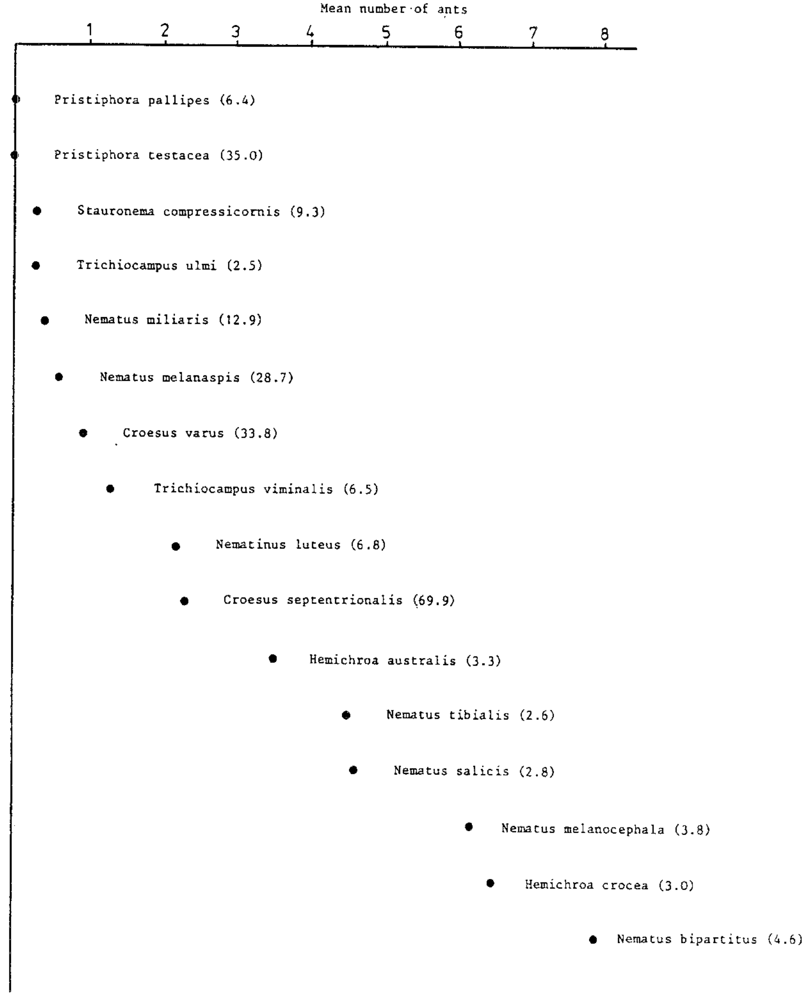


Fig. 6. Attack by ants (mean number of ants during test) and gland size (in parentheses, glandular surface in $10^{-2}$ $mm^2$)

between glandular development and number of ants attacking the larvae ($rs = -0.43$, NS Spearman rank correlation coefficient). This suggests that alternative or additional defensive mechanisms exist.

Two additional experiments proved the efficiency of the secretion: (1) Two larvae of *Nematus melanaspis* were given in succession to the ants. The glands of the first larva had been emptied the previous day. The mean number of ants surrounding the larva with emptied glands was 10.2, whereas it was only 1.7 for the second larva. (2) *Myrmica rubra* workers dispersed when presented filter

papers impregnated with the secretion of *Croesus septentrionalis, C. varus, Nematinus luteus, Nematus melanaspis, N. pavidus, N. spiraeae, Pristiphora erichsonii, P. pallipes*, or *P. testacea*. The ants always avoid these papers and often show signs of excitement or discomfort.

Among the alternative defensive mechanisms, the dense pubescence of *Trichiocampus* larvae seems to deter efficiently the ants. If, however, an ant does succeed in biting a *T. viminalis* larva, the larva quickly drops from its leaf, thus escaping from further attack.

A very different behavior was observed in *Nematus melanaspis, N. miliaris*, and *Croesus varus*. Those larvae grip the leaf firmly with their thoracic legs and are able to dislodge biting ants by movements of the abdomen. *Nematus melanocephala* raises and moves its abdomen without releasing any secretion. In this species defensive behavior is thus purely mechanical and only effective when the ants are few.

*Nematinus luteus* seldom raises its abdomen, but releases a secretion smelling strongly of citral when disturbed (Boevé et al., 1984). For these larvae immobility is an efficient defensive reaction: ants usually step on them without noticing them. If, however, a larva is bitten, movements of the abdomen and emission of the secretion repel the ants.

A remarkable defensive mechanism is observed in *Stauronema compressicornis*. The larva is well known to build foam pillars (with saliva?) on both sides of the leaf, completely surrounding the insect (Figure 7). When an ant approaches, the larva moves to the opposite side of the leaf, the ant hits the pillars and immediately retreats rubbing its antennae on the substrate. Larvae separated from their leaf and pillars still seem to deter the ants by producing a sticky secretion, the nature and origin of which need further studies.

*Palatability to Great Tits*. Only a few larvae were rejected by the birds, which otherwise accepted the sawfly larvae either as readily as control mealworms or with various signs of reluctance or discomfort. The great tits eat some larvae only after long delay. Those larvae were tested and rejected several times before being swallowed, or eaten piece by piece, whereas the mealworms were always eaten entirely at once. When swallowing a larva, a bird sometimes rubbed its bill on the substrate and/or ruffled its feathers. In all experiments, the mealworm given at the end was eaten without hesitation.

The results of these experiments are given in Table 2. Even though the number of repetitions was small, some clear conclusions can be drawn. On the one hand, the birds readily ate most cryptic larvae in the same way as mealworms, although some of these larvae possess well-developed glands and secretion. Larvae of *Croesus varus* were the only cryptic ones eaten with some reluctance, and this will be discussed later. On the other hand, the birds always showed various degrees of reluctance to eat aposematic larvae, even those with extremely reduced ventral glands. The proportions of fully palatable larvae are

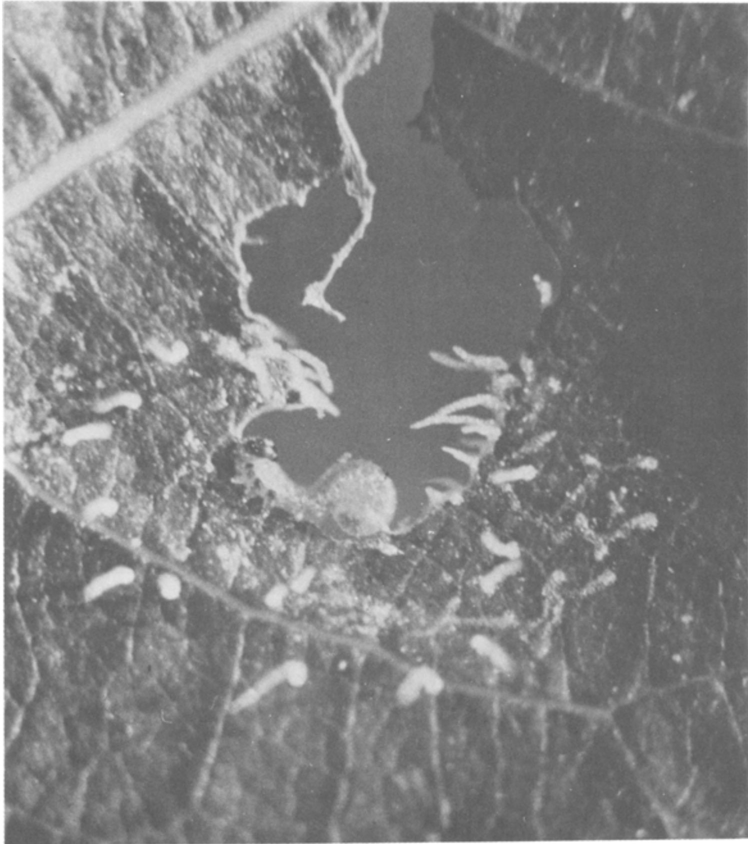

Fig. 7. A larva of *Stauronema compressicornis* surrounded by spumous pillars.

much higher in cryptic than in aposematic species, and this difference is highly significant ($P < 0.001$, Fisher's exact probability test for two samples). This indicates that bright colors do indeed have an aposematic function.

In most cases, aposematic larvae were, however, eventually swallowed, and we never observed any later sign of discomfort or poisoning in the birds. Neither did we observe, during this experimentation, any long-lasting avoidance conditioning to those larvae. Most aposematic sawfly species were thus only moderately distasteful and apparently not toxic to great tits, even though some larvae were eventually rejected. The distributions of gland sizes are not significantly different in palatable and unpalatable species ($P \simeq 0.15$, median test). However, the Fisher test suggests a correlation between voluminous glands and unpalatability: the proportion of species with voluminous glands ($>0.2$ mm$^2$) is higher in unpalatable species ($P \simeq 0.036$). The defensive secretion from ventral glands

TABLE 2. PALATABILITY OF NEMATINE LARVAE TO TWO GREAT TITS (I, II)[a]

| | Appearance | Glandular development (glandular surface in $10^{-2}$ mm$^2$) | Response of the birds I | Response of the birds II |
|---|---|---|---|---|
| *Croesus septentrionalis* | A | 86.4 | –(b, c) | –(a) |
| *C. varus* | C | 33.8 | –(b, c, d) | –(c, d) |
| *Hemichroa australis* | C | 3.3 | + | + |
| *H. crocea* | A | 3.3 | –(b, c, d) | |
| *Nematinus luteus* | C | 6.8 | + | |
| *Nematus bipartitus* | C | 4.6 | + | + |
| *N. melanaspis* | A | 28.7 | –(b, d) | |
| *N. melanocephala* | A | 3.8 | –(b, c, d) | –(b, c, d) |
| *N. miliaris* | A | 15.5 | –(d) | –(b, c, d, then a) |
| *N. pavidus* | A | 40.6 | –(b, d) | –(b, c, d) |
| *N. spiraeae* | C | 36.2 | + | + |
| *N. tibialis* | C | 2.6 | + | + |
| *Pachynematus scutellatus* | C | 7.6 | + | |
| *Pontania viminalis** | ? | 1.5 | + | |
| *Pristiphora compressa* | C | 11.5 | + | |
| *P. pallipes* | C | 6.4 | + | + |
| *P. saxesenii* | C | 16.4 | + | |
| *P. testacea* | A | 35.0 | –(b, c, d, then a) | |
| *Stauronema compressicornis* | C | 9.3 | | + |
| *Trichiocampus viminalis* | A | 6.5 | –(b, c, d) | –(a) |

[a]+: accepted without reluctance; –: various signs of reluctance shown as specified in parentheses; a: the larva is pecked and eventually rejected; b: the larva is tested several times and eaten after a long delay, often piece by piece; c: the bird rubbed its bill on the substrate; d: the bird ruffled its feathers; * galls were given to the bird and were opened by it; A: aposematic; C: cryptic.

can be effective against great tits but only when it is produced in large quantity. Larvae of *Croesus septentrionalis* became more palatable and those of *C. varus* completely palatable, after their secretions were first experimentally removed. Both species secrete the pentacyclic monoterpene, dolichodial (Boevé et al., 1984). They are vigorous species with well-developed ventral glands. Interestingly, *Nematus spiraeae* has glands of about the same size as those of *C. varus*, and also produces dolichodial as a major constituent of the secretion (Boevé et al., 1984). *N. spiraeae*, however, was readily accepted by the birds. We have no straightforward explanation for these contradictory results, which could be due to the small number of tests performed. Also, the size of the gland is only a rough approximation of the amount of secretion produced.

## DISCUSSION

Defensive mechanisms are very diversified in larval nematines. The ventral gland secretion is just one of them, prominent in some species, absent in many others. The occurrence of ventral glands is a common feature to nearly all nematines. Only the non-European species *Pikonema alaskensis* is devoid of them (Maxwell, 1955). No other function than defense is known for the secretion of these glands. This chemical defense has thus been secondarily lost in species with reduced, apparently nonfunctional glands. An alternative hypothesis is that small glands could produce minute amounts of very toxic compounds. Up to now there are no data supporting this hypothesis, which seems to us unlikely. Indeed, no secretion at all could be collected from reduced glands, and we do not know of any insect protected only by trace compounds. Although, as demonstrated in this study, it can be effective against some predators, ventral gland secretion must have its drawbacks.

We do not know which selective pressures could favor alternative modes of defense. Important factors such as the cost of chemical defense or of the alternative mechanical defenses, e.g., pubescence of *Trichiocampus* larvae or the spumous pillars built by *Stauronema compressicornis*, have not yet been studied. Counteradaptation of predators to the secretion could lead to the development of new modes of defense. Again this aspect of defense remains little studied. It has been reported that some parasitoids are attracted instead of deterred by the defensive movements of the larvae of the sawfly, *Neodiprion swainei*, which, however, belongs to another family (Diprionidae) and is devoid of ventral glands (Tripp, 1962).

Sympatric bright-colored species of similar appearance, some with well-developed glands, others with reduced glands, could be interpreted as a Batesian mimicry complex as suggested earlier (Pasteels, 1976). Our study cannot confirm or reject this hypothesis. Aposematic species with reduced glands were eaten only with reluctance by the birds. We cannot specify if this is due to some innate or learned response to aposematic colors or if chemical defenses other than the ventral gland secretion are present in these species.

The constituents of the ventral glands are diverse. They include aliphatic alcohols, aldehydes, and acetates; benzaldehyde; and various monoterpenes, cyclic or not (Boevé et al., 1984, Bergström et al., 1984). These are well-known insect defensive compounds, and their efficiency against ants was confirmed by our results. In contrast, these secretions seem less efficient against birds. Tables 3 and 4 summarize the main results which suggest a higher efficiency against ants. Many species with functional glands were readily accepted by great tits. It is, of course, difficult to draw general conclusions from experiments using only two of the potential predators. We have, however, no reason to suspect that

TABLE 3. COMPARISONS OF GLAND SIZE DISTRIBUTIONS IN SAWFLY LARVAE: (MEDIAN TEST)

| Comparison | Result |
|---|---|
| Cryptic versus aposematic | NS, $P < 0.23$ |
| Heavily attacked by ants versus less attacked | Highly significant, $P < 0.005$ |
| Palatable versus unpalatable to great tits | NS, $P \simeq 0.15$ |

great tits, of all the insectivorous birds, or *Myrmica rubra*, of all the predacious ants, have developed exceptional reactions towards sawfly larvae. Our results thus suggest that the secretion of the ventral glands is aiming primarily at insect predators (e.g., ants) and not at birds. This supports the hypothesis that volatile irritants commonly found in insect secretions have evolved against small arthropod enemies (Pasteels et al., 1983). Nematine secretion needs to be produced in large quantity to show some activity against birds. The proportion of larvae with very large glands is significantly higher in aposematic species than in cryptic species.

All brightly colored species were proved to be slightly to highly unpalatable for great tits, which suggests that bright coloration has an aposematic function. These larvae, however, did not appear to be highly toxic, and it has been reported that brightly colored *Nematus ribesii* larvae were captured in large quantities by cuckoos (Coward, 1920), and *Croesus septentrionalis* larvae by young chaffinches (Benson, 1950).

We do not believe that such occasional observations demonstrate that these larvae are not protected. Even a slight unpalatability could be sufficient to lower predation in complex communities, especially when more favorable food is available. Also, if no conditioning of the birds to unpalatable larvae was observed during the experimentation when few larvae were tested at one time, this does not preclude that conditioning to gregarious larvae could occur in nature.

Aposematic colors are linked to gregarious habit. This is a common feature in insects (Fisher, 1930). Gregariousness increases the efficiency of the signal and is not compatible with crypsis. Altruistic behavior, i.e., the warning of predators, is more expected to evolve by kin selection in aggregated siblings issued from a single egg batch (ref. in Pasteels et al., 1983).

TABLE 4. CORRELATIONS CONFIRMED BY FISHER'S TEST

| Correlation | P |
|---|---|
| Aposematism and voluminous glands ($>0.1$ mm$^2$) | $P \simeq 0.046$ |
| Protection against ants and medium-sized or voluminous glands ($>0.05$ mm$^2$) | $P < 0.001$ |
| Unpalatability to great tits and voluminous glands ($>0.2$ mm$^2$) | $P \simeq 0.036$ |

Only two classes of predators were tested against sawfly larvae. Their known enemies are, however, very diverse (Benson, 1950). Among them, parasitoids are of prime importance. Any attempt to study the evolution of defensive strategies in sawflies should take their role into account. Studies on defense against parasitoids are badly needed.

Sawfly species with completely different defensive strategies are often found on the same host plant. For example, the cryptic solitary *Stauronema compressicornis* surrounded by defensive pillars; the gregarious, aposematic, and hairy *Trichiocampus viminalis* with reduced ventral glands; and the aposematic *Nematus melanaspis* with copious defensive secretion, can be found on the same *Populus* tree. In a complex environment, a given set of selective pressures does not necessarily lead to the evolution of a single optimal solution, but rather to a finite number of stable equilibrium.

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# ANAL GLAND SECRETIONS OF THE STOAT (*Mustela erminea*) AND THE FERRET (*Mustela putorius* FORMA *furo*) Some Additional Thietane Components

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**Abstract**—Two new thietanes, *cis-* and *trans*-2-ethyl-3-methylthietane, have been identified in the anal gland secretion of the stoat, and 2-isopropyl thietane has been characterized from the anal gland secretion of the ferret. Conflicting published data are reevaluated.

**Key Words**—Anal gland secretion, mustelid, ferret, stoat, thietane.

## INTRODUCTION

A number of studies of the components of the anal gland secretions of some mustelid species have recently been published (Crump, 1980a,b; Brinck et al., 1983; Schildknecht et al., 1981; Schildnecht and Birkner, 1983; Schildnecht and Hiller, 1984). These chemical analyses have confirmed (Brinck et al., 1983) the phylogenetic relationships at the generic level within the family Mustelidae. The anal gland secretions of species within the genus *Mustela* contain thietanes and dithiolanes, compounds which have not been found in the genera *Meles, Martes,* or *Lutra*. However, illumination of the relationships within the genus *Mustela* is difficult, as arguments based merely on the presence or absence of components are not particularly helpful. For example, compare the definitive report on the anal gland secretion from mink (*Mustela vison*) (Schildknecht et al., 1981), in which 11 sulfur-containing compounds were identified, with that of Brinck et al. (1983), where only three were noted.



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In this paper some new minor thietanes are identified in a reexamination of the anal gland secretions of the stoat (*Mustela erminea*) and the ferret (*Mustela putorius* forma *furo*).

## METHODS AND MATERIALS

Proton magnetic resonance spectra ([$^1$H]NMR) were obtained on Varian XL200, FT80A, or EM360 instruments. A Shimadzu QP1000 combined gas chromatograph–mass spectrometer and data system fitted with a 50-m BPI fused silica capillary column was used for GC-MS analysis. A Varian 2700 gas chromatograph fitted with a 2-m × 3-mm 5% OV-101 column was used for preparative GLC, and a Pye GCD fitted with a 25-m BPI fused silica capillary column was used for analytical GLC.

Stoats were trapped as reported by Crump (1980a), and the diethyl ether extracts of the anal gland secretions were analyzed by capillary GLC and GC-MS. Anal gland secretions were obtained from live male and female ferrets at Kewai Farms Ltd., Marlborough, New Zealand. These ferrets were of Swedish and Finnish origin and had been imported from Finland in 1983. Samples were also obtained from the first generation of these animals produced in New Zealand. The anal gland secretions were treated as described previously (Crump, 1980b) and were analyzed by capillary GLC and GC-MS.

The synthetic thietanes and dithiolanes used in this work that had been identified in earlier studies were prepared as previously described (Crump, 1980a,b, 1982).

*2-Ethyl-3-methylthietanes*. The 2-ethyl-3-methylthietanes were prepared from 2-methylpentane-1,3-diol via the carbonate ester as described by Crump (1982) for the synthesis of (2*S*)-2-propylthietane. The isomers were separated by preparative GC to yield a lower boiling component (isomer a) and a higher boiling component (isomer b) (relative stereochemistry not assigned). Retention times relative to 2-propylthietane: 0.81 (isomer a) and 0.93 (isomer b). Mass spectrum (essentially identical for both isomers): 116(41), 101(10), 74(100), 55(41), 41(86). [$^1$H]NMR $\delta$ $CDCl_3$) isomer a: 0.85 (t, $J$ = 7 Hz, 3H, ethyl $CH_3$), 1.12 (d, $J$ = 7 Hz, 3H, methyl $CH_3$), 1.74 (m, 2H, ethyl $CH_2$), 2.85 (m, 1H, $H_4$), 2.94 (m, 1H, $H_3$), 3.02 (m, 1H, $H_4'$), 3.32 (m, 1H, $H_2$). [$^1$H]NMR ($CDCl_3$) isomer b: 0.87 (t, $J$ = 7 Hz, 3H, ethyl $CH_3$), 1.16 (d, $J$ = 7 Hz, 3H, methyl $CH_3$), 1.74 (m, 2H, ethyl $CH_2$), 2.71 (m, 1H, $H_4$), 3.28 (m, 1H, $H_4'$), 3.90 (m, 1H, $H_3$), 4.12 (m, 1H, $H_2$).

*2-Isopropylthietane*. 2-Isopropylthietane was prepared from 4-methylpentane-1,3-diol via the bis(benzenesulfonate) method of Buza et al. (1978). The product was purified by preparative GC to give 2-isopropylthietane as a colorless liquid. Mass spectrum: 116(100), 101(94), 73(98), 69(45), 55(42), 45(47). [$^1$H]NMR ($CDCl_3$) 0.83 (d, 6H, $J$ = 7 Hz, *gem* $CH_3$), 1.82 (m, 1H, isopropyl CH), 2.75 (m, 2H, $H_3$ and $H_3'$), 3.10 (m, 2H, $H_4$ and $H_4'$), 3.42 (m, 1H, $H_2$).

## RESULTS AND DISCUSSION

*Stoat* (Mustela erminea). The compounds characterized by Crump (1980a) in the anal gland secretion of the stoat were 2-ethylthietane (2), 2-propylthietane (5), 2-pentylthietane (7), 3-ethyl-1,2-dithiolane (6), 3-propyl-1,2-dithiolane (8), and indole (9) (see Scheme 1). Using capillary GC, Brinck et al. (1983) confirmed these results and also reported the presence of 2,2-dimethyl-thietane (11), *trans*-2,3-dimethylthietane (1) and *o*-aminoacetophenone (10). In addition, two unidentified compounds that were concluded to be isomers of 2-propylthietane (5) were detected. Schildknecht and Birkner (1983) also reinvestigated the secretion of the stoat, confirming the results of Crump (1980a) and reporting the presence of 2-methylthietane and one of the isomers of 2,3-dimethylthietane.

SCHEME 1.

Reexamination of the stoat secretion using capillary GC and GC-MS was carried out to characterize the two unidentified compounds noted by Brinck et al. (1983). In the gas chromatogram of the stoat extract (Figure 1), compounds 1, 2, 5–10 have been characterized previously by GC and GC-MS comparisons of the relative retention times and mass spectra of authentic compounds (Crump, 1980a; Brinck et al., 1983).

*Compounds 3 and 4*. For compound 3: mass spectrum 116(32), 101(6), 87(6), 74(100), 69(21), 55(28), 41(55). The molecular weight is consistent with the formula $C_6H_{12}S$, which indicates an isomer of 2-propylthietane. Loss of methyl (*m/e* 101) ethyl (*m/e* 87), and a base peak *m/e* 74 ($M^+$-$CH_3CHCH_2$) imply that this isomer is 2-ethyl-3-methylthietane. This assignment was con-

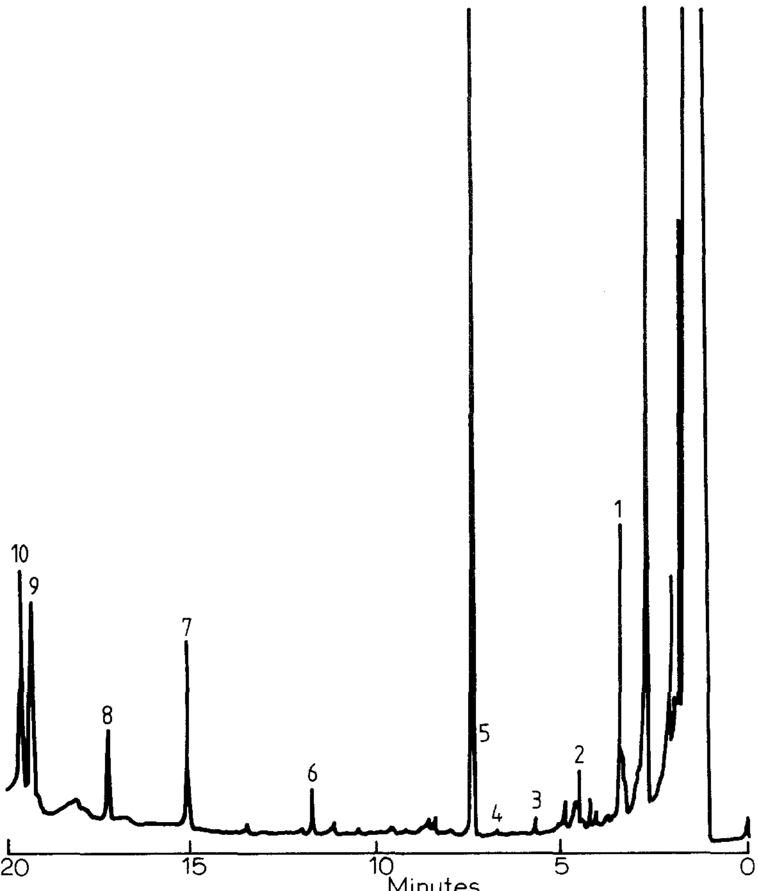


FIG. 1. Gas chromatogram of stoat anal gland extract (fused silica capillary column BPI, 25 m, 60° 1 min; 4°/min to 150°; 1 cm/min).

firmed by comparison of the mass spectrum with those of authentic *cis*- and *trans*-2-ethyl-3-methylthietane. The identity of compounds 3 and 4 was further established by peak enhancement of these compounds by coinjection of the stoat extract and the synthesized isomeric compounds. The relative stereochemistry of the ethyl and methyl groups in the isomeric 2-ethyl-3-methylthietanes could not be established from [$^1$H]NMR studies (2DJ spectroscopy) because neither isomer exhibited $H_2$-$H_3$ coupling. Therefore, compounds 3 and 4 are the *trans*- and *cis*-2-ethyl-3-methylthietanes, but the relative stereochemistry has not been determined.

*Ferret* (Mustela putorius *forma* furo). The compounds characterized (GC-MS and GC comparison with authentic material) in the anal gland secretion of

the ferret by Crump (1980b) were 2,2-dimethyl-thietane (11), *trans*-2,3-dimethylthietane (1), *cis*-2,3-dimethylthietane (12), 2-propylthietane (5), 3,3-dimethyl-1,2-dithiolane (14), *trans*-3,4-dimethyl-1,2-dithiolane (15), *cis*-3,4-dimethyl-1,2-dithiolane (16), 2-pentylthietane (7), 3-propyl-1,2-dithiolane (8), quinoline, and indole (9). These results were substantiated by Schildknecht and Birkner (1983) in their GC-MS analysis of the ferret. The dithiolane components of the secretion were not characterized because the mass spectra of these compounds do not reveal helpful structural information because loss of the sulfur atoms is the principal fragmentation.

Schildknecht and Birkner (1983) also examined the anal gland secretion of the polecat (*Mustela putorius*), and they found essentially the identical pattern of thietanes and dithiolanes which they had detected in the ferret. In contrast, Brinck et al. (1983) reported a markedly different analysis for the polecat extract. They discussed four components (*trans*-2,3-dimethylthietane, an isomer of 2-propylthietane, 3,3-dimethyl-1,2-dithiolane, and indole) in their gas chromatogram and reported that the other peaks were due to solvents or artifacts. However, when compared with those in their mink and stoat chromatograms, the relative retention times of the first group of major components in their gas chromatogram of the polecat (Brinck et al., 1983, their Figure 2F) suggest that these compounds could in fact be 2,2-dimethylthietane, *trans*-2,3-dimethylthietane, and *cis*-2,3-dimethylthietane. If this is correct, the analysis of Brinck et al. (1983) would agree with that of Schildknecht and Birkner (1983).

In an attempt to clarify these results, we examined samples of anal gland secretions from Scandinavian ferrets, these being the animals available in New Zealand which most closely resemble the Swedish polecats studied by Brinck et al. (1983). The ferret is probably a domesticated form of the polecat since the two are interfertile and have identical karyotypes (Volobuev et al., 1974). A typical gas chromatogram of our extracts is presented in Figure 2. There is some variation between individuals in the intensity of the components, but the overall pattern is essentially as shown. The compounds are the same as those identified in the previous studies of the ferret (Crump, 1980b; Schildknecht and Birkner, 1983) with the addition of compounds 2 and 13. We could not detect the isomer of 2-propylthietane which Brinck et al. (1983) found in the polecat. Compound 2 was shown to be 2-ethylthietane by comparison of the mass spectrum and GC retention time with authentic material.

*Compound 13*. Mass spectrum 116(74), 101(100), 73(74), 70(42), 69(68), 55(62), 45(83). Analysis of the mass spectrum implied that compound 13 was another isomer of 2-propylthietane not previously found. The loss of methyl (*m/e* 101) and propyl (*m/e* 73) indicated that the structure was 2-isopropylthietane. This was shown to be correct by comparison with the mass spectrum and retention time on GC of authentic 2-isopropylthietane.

We agree with Schildknecht and Birkner (1983) that ferrets and polecats

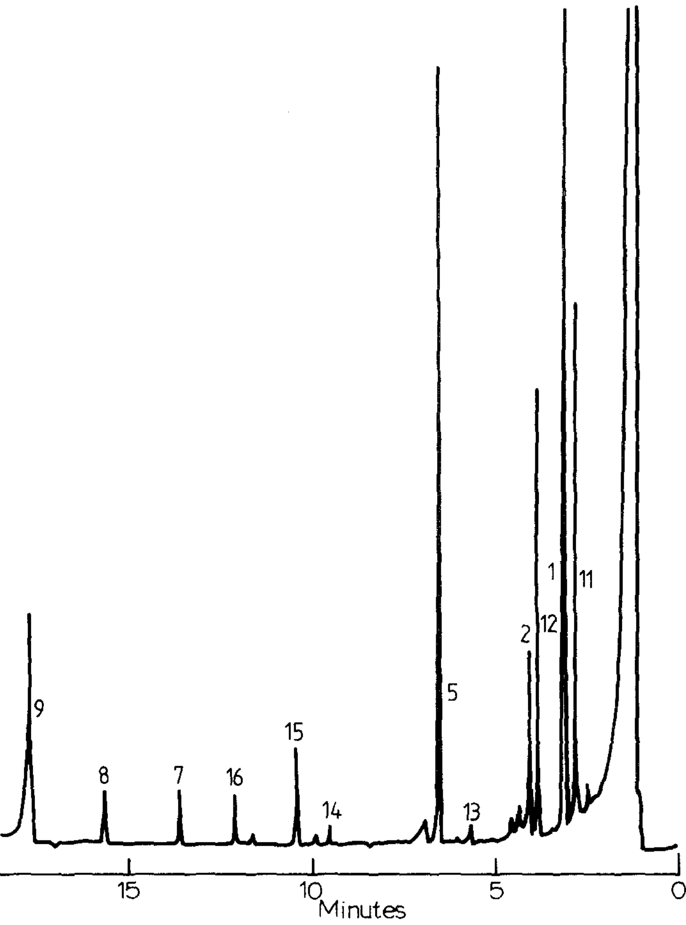


FIG. 2. Gas chromatogram of ferret anal gland extract (fused silica capillary column BPI, 25 m, 60° 1 min; 4°/min to 150°; 1 cm/min).

have secretions with similar compositions, in contrast to the conclusions of Brinck et al. (1983).

This report has characterized further examples of the wide range of thietanes and dithiolanes that are elaborated by the Mustelidae in their anal gland secretions. Some additional minor thietanes, probably structural isomers of 2-propylthietane, were noted by Schildknecht and Birkner (1983) in their analysis of the anal gland secretion of the weasel (*Mustela nivalis*). However, only the molecular weights of these compounds were reported. Since they have similar relative retention times, we consider that these compounds are likely to be the isomers identified in this paper.

Although those members of the genus *Mustela* that have been investigated

produce a range of structurally isomeric thietanes and dithiolanes, the principal components in the anal gland secretions serve effectively to differentiate the species. Thus the major thietanes are 11, 1, 12, 5 in the ferret and polecat, 5 in the stoat, 11 in the mink, and 1 (Shildknecht and Birkner, 1983) in the weasel. Our current studies are directed towards the inter- and intraspecific uses of these semiochemicals by the Mustelidae and other species.

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# FEEDING DETERRENCY OF SOME PYRROLIZIDINE, INDOLIZIDINE, AND QUINOLIZIDINE ALKALOIDS TOWARDS PEA APHID (*Acyrthosiphon pisum*) AND EVIDENCE FOR PHLOEM TRANSPORT OF INDOLIZIDINE ALKALOID SWAINSONINE

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**Abstract**—The feeding deterrency of a series of pyrrolizidine, indolizidine, and quinolizidine alkaloids and selected derivatives was measured against the pea aphid (*Acyrthosiphon pisum* Harris). The indolizidine alkaloid, castanospermine, was intensely active ($ED_{50}$, 20 ppm) as were the quinolizidine alkaloids, but only modest feeding deterrency was observed with most of the pyrrolizidine alkaloids tested. The insect survival rate of aphids on a castanospermine-supplemented diet over 24 hr was also very low relative to the controls. Castanospermine does not inhibit aphid trehalase. The indolizidine alkaloid swainsonine occurred in the honeydew of pea aphid feeding on the locoweed, *Astragalus lentiginosus*. Since the pea aphid is a phloem feeder, swainsonine must be transported in the phloem.

**Key Words**—Feeding deterrents, *Acyrthosiphon pisum*, Homoptera, Aphididae, host-plant resistance, pyrrolizidine, indolizidine, quinolizidine, alkaloids, locoweed, honeydew, *Astragalus lentiginosus*, phloem transport.

## INTRODUCTION

Pyrrolizidine, indolizidine, and quinolizidine alkaloids share the common structural features of a bicyclic ring system with a bridgehead nitrogen. Botanically, such alkaloids are often associated with legumes, and in certain legumes some of these alkaloids appear to play a role in mediating the adaption of insects to their host. Thus, pyrrolizidine alkaloids are known to play a key role in host-



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plant selection and as a sex pheromone in certain danaid butterflies (Jones and Blum, 1983).

If such alkaloids are transported in the phloem to any degree, they could play an especially important role in host-plant relationships of sapfeeding insects, for example aphids. One case has been reported (Smith, 1966) where sparteine, a quinolizidine alkaloid, acts as a feeding stimulant for the aphid *Acyrthosiphon spartii*. Another aphid, *Aphis cytisorum*, prefers low quinolizidine alkaloid-containing plants but nevertheless has the ability to sequester and metabolize quinolizidine alkaloids (Wink et al., 1982). Quinolizidine alkaloids have also been shown to provide host-plant protection against the pea aphid (*Acyrthosiphon pisum*) in lupines (Wegorek and Krzymanska, 1970).

Among the sap-feeding aphids found on legumes, the pea aphid can be considered paradigmatic. In order to explore structure–activity relationships, representative members of these alkaloids were tested against the pea aphid employing a standard aphid bioassay designed to measure feeding deterrency (Dreyer et al., 1981).

## METHODS AND MATERIALS

*Bioassay.* Bioassays were performed by adding a series of known concentrations of alkaloids to standard Akey diet at pH 7.0 (Akey and Beck, 1971). The 24-hr bioassay was run following procedures previously described (Dreyer et al., 1981). The $ED_{50}$ (effective dose, at which half the aphids were feeding) values were obtained from the dose–response curve.

*Inhibition Studies.* Trehalase activity was determined by measuring reducing end groups using Flozyme® reagent (Worthington Biochemical). The supernatant from grinding 1 g of aphids with 5 ml cold acetate buffer, pH 5.6, was incubated with trehalose and castanospermine added at a series of concentrations along with the appropriate controls. Flozyme reagent was then added and adsorption measured at 420 nm. Values for the alkaloid-containing runs did not differ significantly from the controls.

*Alkaloids.* Castanospermine (V) was isolated from *Castanospermum australe* seeds and swainsonine (IV) (Molyneux and James, 1982) from *Astragalus lentiginosus* using the entire plant (Figure 1).

Riddelliine was isolated from *Seneco riddellii*, seneciphylline from *S. douglasii* var. *longilobus*, and senecionine from *S. triangularis*. Monocrotaline was isolated from *Crotalaria spectabilis*. The above alkaloids were characterized by melting points, mass spectra [$^{1}$H]- and [$^{13}$C]NMR spectroscopy (Molyneux et al., 1979, 1981).

The N-oxides were prepared from the alkaloids by treatment with 30% $H_2O_2$ in $CHCl_3$–EtOH according to published procedures (Culvenor et al., 1970).

Retronecine was prepared by hydrolysis of riddelliine with $Ba(OH)_2$ (Culvenor and Smith, 1955). The lupine alkaloids tested were commercial samples.

Deoxymannonojirimycin (I)  Nojirimycin (II)  Deoxynojirimycin (III)

Swainsonine (IV)  Castanospermine (V)

Seneciphylline, R=H
Riddelliine, R=OH

Senecionine

Monocrotaline

Retronecine

Lupinine  Cytisine  Sparteine

FIG. 1. Structures of polyhydroxypiperidines, indolizidine, and pyrrolizidine alkaloids.

TABLE 1. FEEDING DETERRENCY OF SELECTED PYRROLIZIDINE, INDOLIZIDINE, AND QUINOLIZIDINE ALKALOIDS AND DERIVATIVES

| | $ED_{50}$ (% of diet) ± SE | |
|---|---|---|
| Alkaloid | Parent alkaloid | Derived N-oxide |
| Pyrrolizidine alkaloids | | |
| Seneciphylline | 0.04 ± 0.006 | |
| Riddelliine | 0.03 ± 0.006 | 0.16 ± 0.04 |
| Senecionine | 0.17 ± 0.05 | 0.1 ± 0.025 |
| Monocrotaline | 0.05 ± 0.03 | inactive at 0.25% |
| Retronecine | >1 | |
| Indolizidine alkaloids | | |
| Castanospermine | 0.00002[a] ± 0.00001 | 0.03 ± 0.003 |
| Castanospermine tetraacetate | 0.07 ± 0.005 | |
| Swainsonine | inactive at 0.05 | |
| Quinolizidine alkaloids | | |
| Lupinine | 0.00082 ± 0.0003 | |
| Cytisine | 0.00016 ± 0.00004 | |
| L-Sparteine | 0.00011 ± 0.00005 | |

[a]Low survival rate.

## RESULTS

The data obtained are displayed in Table 1 and expressed in $ED_{50}$ values, the concentration of the test substance in a synthetic diet at which half the aphids will feed.

The feeding deterrency of the pyrrolizidine alkaloids was significant but not impressive. For the most part, the derived N-oxides were less active than the parent alkaloids. Most common naturally occurring pyrrolizidine alkaloids are macrocyclic diesters (Bull et al., 1968). Deesterification of those diesters gives the necine base, retronecine. The low activity of retronecine suggests that the macrocyclic necic acid moiety is required for biological activity.

Most striking were the results with castanospermine (V), which was several orders of magnitude more active than the most active substance previously tested in this program (Rose et al., 1981), but more importantly, the insect survival rate on castanospermine supplemented diets was very low. Conversion of castanospermine to its tetraacetate or the N-oxide resulted in a large decrease in feeding deterrency. The other indolizidine alkaloid tested, swainsonine (VI), a known α-mannosidase inhibitor (Dorling et al., 1980), showed no activity as a feeding deterrent when added to a synthetic diet at 0.05%.

The mechanism of action of such polyhydroxyindolizidines toward the pea aphid is most likely different from the pyrrolizidine alkaloids and is probably

due to their known ability to inhibit hydrolases. Such carbohydrase inhibition is associated with a series of structurally related polyhydroxypiperidine (I–III) (Murao and Miyata, 1980; Niwa et al., 1970; Reese et al., 1971) and indolizidine alkaloids (IV, V) (Dorling et al., 1980; Saul et al., 1983; Tulsiani et al., 1982). These alkaloids show high selectivity towards the enzymes catalyzing the hydrolysis of various glycosides. Swainsonine (IV) is an $\alpha$-mannosidase inhibitor (Dorling et al., 1980), while the closely related castanospermine (V) can inhibit both $\beta$- and some $\alpha$-glucosidases, depending on the enzyme origin (Saul et al., 1983). In view of the trehalase inhibition exhibited by 1,5-dideoxy-1,5-imino-D-mannitol (I) (Murao and Miyata, 1980) and the structural similarities between the monocyclic polyhydroxypiperidines (I–III) and theh bicyclic indolizidine alkaloids, IV and V, it appeared to be of particular interest to investigate the inhibitory effect of the latter towards this enzyme also.

The nonreducing disaccharide, trehalose, is a key transport and storage sugar in many, if not all, insect species. Trehalase, the enzyme which converts trehalose to glucose, occurs in the gut, hemolymph, and muscle of insects. Trehalase from the insect gut differs greatly in its properties (pH optimum, electrophoretic mobility, heat stability, $K_m$, solubility, elution profiles, etc.) from trehalase in insect muscle. Since there is little or no trehalose in the diet of most insects, the role of trehalase in the gut is not clear. On the other hand, trehalase in the hemolymph and muscle serves to convert trehalose to glucose during energy mobilization (Candy and Kilby, 1975).

This role played by trehalase in insect energy mobilization does not have a parallel in mammals. As a result, specific trehalase inhibitors may offer a means of selective insect control that would have minimal impact on mammals. The dramatic results obtained with castanospermine at very low concentrations suggested that it could be specifically inhibiting trehalase. However, when tested against the enzyme fraction isolated from the pea aphid, it had no effect on the hydrolysis rate of trehalose. As a result, the likely effect of castanospermine is due to its known ability to inhibit $\alpha$-glucosidase (Saul et al., 1983).

The pea aphid is known to be a phloem feeder and to probe largely intercellularly (Pollard, 1973; McLean and Kinsey, 1967). This mode of probing avoids many potentially toxic substances, especially phenolics which normally are compartmentalized inside cells (Matile, 1984). Since the rate of aphid probing, and hence its success on a plant, depends on its ability to depolymerize intercellular pectin with a salivary pectinase this strategy avoids phenolic pectinase inhibitors (Dreyer and Campbell, 1983; Campbell and Dreyer, 1985).

During the course of this work, it was found that the pea aphid will opportunistically colonize the locoweed, *Astragalus lentiginosus*. TLC examination of honeydew collected from such an *Astragalus* colony showed the presence of substantial amounts of swainsonine (IV). Since the pea aphid is a phloem feeder, swainsonine, which is very water soluble, must have been transported in the phloem and subsequently excreted by the aphid.

Only one other case has been reported which directly demonstrates the phloem transport of alkaloids. This is the recent report of Wink and Witte (1984) on several *Lupinus* spp. Depending on the plant part, the quinolizidine alkaloid content of lupine phloem is 1-5 mg/ml (Wink and Witte, 1984). This is a factor of 10 greater than the $ED_{50}$ values for the quinolizidine alkaloids tested in this study. Hence, some lupines appear to contain considerably more alkaloid in the target tissues than that required to deter aphid feeding. Pyrrolizidine alkaloids can also reach exceptionally high levels in *Senecio* and *Crotalaria* spp. (Molyneux and Johnson, 1984; Molyneux et al., 1979; Johnson et al., 1985) so that they could be effective deterrents in vivo even though not very active in the test system. However, until more is known about the alkaloid distribution in different plant parts and insect pests, statements regarding their ecological role are little more than speculation.

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# AGGREGATION PHEROMONES OF THE FLAT GRAIN BEETLE, *Cryptolestes pusillus* (COLEOPTERA: CUCUJIDAE)[1]

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**Abstract**—Aggregation pheromones were isolated from *Cryptolestes pusillus* (Schönherr), a coleopteran pest of stored products. Porapak Q-captured beetle and frass volatiles were fractionated by preparative gas-liquid chromatography. The fractions were bioassayed with an arena olfactometer and/or with a two-choice, pitfall olfactometer. Three biologically active, male-produced compounds eliciting aggregation behavior from adult *C. pusillus* were isolated and identified by spectroscopic methods as (Z)-3-dodecenolide (I), (Z)-5-tetradecen-13-olide (II), and (Z,Z)-3,6-dodecadienolide (III). Compound I was the major volatile produced and was active alone. Compound II was not active alone, but synergized the response to I. Compound III was active alone at higher concentrations, but did not significantly increase the response when added to the most active mixture of I and II, and so it is probably not part of the aggregation pheromone. Pheromone production increased dramatically when the insects were aerated on a food source.

**Key Words**—*Cryptolestes pusillus*, coleoptera, cucujidae, flat grain beetle, aggregation pheromone, macrolide, (Z)-3-dodecenolide, (Z)-5-tetradecen-13-olide, (Z,Z)-3,6-dodecadienolide.

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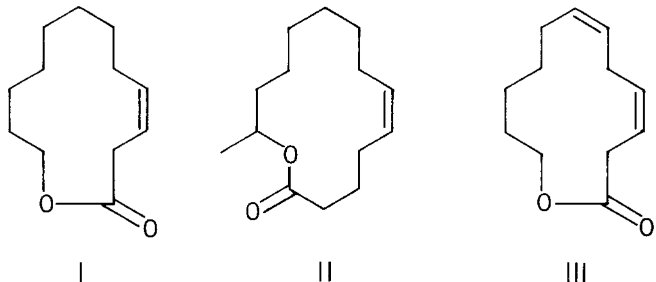
## INTRODUCTION

The flat grain beetle, *Cryptolestes pusillus* (Schönherr), is a serious, worldwide pest of diverse stored products (Reid, 1942; Payne, 1946; Howe and Lefkovitch, 1957). In the United States and Canada, *C. pusillus* is of major importance, especially in the grain-producing Midwestern states (Barak and Harein, 1981; Mueller, 1982). A preliminary study (Quaife, 1980) had indicated that male flat grain beetles produced an aggregation pheromone. Adults of both sexes responded positively to Porapak Q-captured male volatiles in two bioassay methods. No attempt, however, was made to isolate or identify the active compounds.

We report here the isolation and identification of three aggregation pheromones (I–III) from *C. pusillus* beetle and frass volatiles. The syntheses of I–III are reported elsewhere (Millar and Oehlschlager, 1984; Millar et al., 1983).




STRUCTURES I, II, and III.

## METHODS AND MATERIALS

### *Biological Methods*

*Insect Rearing. C. pusillus* were reared on rolled oats and brewer's yeast (95:5, w/w) in 3.8-liter wide-mouthed glass jars. The cultures were maintained at 30° ± 2°C and >60% relative humidity in darkness with a density of 1000–3000 insects/kg of diet. Numbers of insects were estimated by weight (≈3200 insects/g).

Insects for bioassay or aeration were sieved from the rolled oat medium with a No. 25 sieve. The residual tailings plus insects were placed in an enamel pan, and the insects were collected by aspiration as they walked away from the tailings. The tailings were then sifted through a 250-$\mu$m sieve to collect the frass (boring dust plus feces), which was stored at −30°C in screw-cap glass jars.

The sex of adult beetles was determined with a method reported by Rilett (1949). Gentle pressure to the abdomens of cold-immobilized insects caused

reversible extrusion of female, but not male, genitalia. This method was more reliable and much easier than assessing differences in antennal length or counting the hind tarsal segments.

*Trapping of Insect and Frass Volatiles.* Insect volatiles were captured on Porapak Q (Byrne et al., 1974; Verigin, 1980; Wong, 1982). New Porapak Q (50/80 mesh) was thermally conditioned at 240°C for 24 hr with helium purge, followed by Soxhlet extraction with ether for 24 hr. The clean Porapak Q was then packed into glass traps (20 × 2.4 cm OD).

Air was drawn by a water aspirator (≈2 liters/min) through a water bubbler and an activated-charcoal scrubber (12 × 2.4 cm OD, 50/80 mesh charcoal) over beetles in a 2-liter Erlenmeyer flask, and through a Porapak Q trap. Batches of 20,000–100,000 insects of mixed age and sex were aerated for a period of three days. They were then allowed to feed for at least three days before being aerated again. Each Porapak Q trap was loaded with up to 50 million beetle-hours (bh) of volatiles (1 bh = the volatiles produced by one beetle in one hour). A smaller version of the aeration apparatus was used to aerate insects of known sex. Purified, moist air was drawn (500 ml/min) over several hundred male or female insects in a 125 ml Erlenmeyer flask, and then through a Porapak Q trap (12 × 1.4 cm ID). For determining the effect of feeding on pheromone production, ≈3,000 insects were aerated on 50 g of rolled oats in a glass chamber (20 × 15 cm O.D.) for a period of one week.

Volatiles were extracted from the Porapak Q by Soxhlet extraction for 24 hr with pentane (doubly distilled in glass). The pentane extracts were concentrated to ≈2 ml with a 30-cm Dufton column, made up to 10.0 ml, and stored in glass vials with Teflon-lined screw caps at −30°C. Pentane-extracted Porapak Q was further extracted with ether, air-dried for several days, repacked into a trap, and heated to 60°C for 8 hr under helium to remove traces of solvent. The ether extract was treated as described for the pentane extract.

For capture of frass volatiles, ≈300 g of frass were packed into a glass tube (40 × 4.8 cm OD). The tube inlet was fitted with a charcoal scrubber, and air was drawn (≈2 liters/min) through the scrubber, the tube of frass, and a Porapak Q trap for a period of one week. The frass was then ground in a ceramic ball mill, and reaerated for another week. The Porapak Q was then extracted as described above. Amounts of frass volatiles were calculated in gram-hours (1 gh = the volatiles from one gram of frass aerated for one hour).

### *Bioassay Procedures*

*Arena Olfactometer.* The arena olfactometer bioassay was as described by Borden et al. (1979) with minor modifications. The arena was a 15 × 15-cm piece of black construction paper clipped to a glass plate, with an air nozzle (flow rate, 800 ml/min) in the middle of one side. A glass tube containing a rolled-up filter paper impregnated with the stimulus was slipped onto the air

nozzle, and the air flow was directed towards the insect release point at the center of the arena.

Insects for bioassay were held in vials in darkness for ≈48 hr without food (15 insects/vial). In later bioassays, the insects were held for 48 hr in a 1-liter flask, with a moist air flow of ≈2 liters/min to remove any insect-produced volatiles. They were then segregated into groups of 15 in vials 1–2 hr before use. In a typical bioassay, a vial of 15 insects was inverted onto the center of the arena and left in place so that the insects could not escape while the test stimulus was being placed in the air stream. Removal of the vial allowed the insects to walk freely. Any insect reaching a 3-cm × 1-cm-wide area in front of the stimulus tube was counted as a positive responder. Any insect reaching the arena edge anywhere else was removed and counted as a nonresponder, as was any insect still walking around on the arena at the end of the allotted 2-min time limit. Dead or maimed insects were subtracted from the number of possible responders.

Each stimulus, including pentane controls, was tested with six replicates of 15 insects each. A fresh stimulus tube was used for each replicate, and the arena paper was changed for each new stimulus. Check runs were also done before each set of bioassays with a standard stimulus solution of a crude extract to ensure that the insects were responding well. On a given day, all test insects were drawn from the same population and each group of insects was used only once. Bioassays were run at room temperature under fluorescent light.

*Two-Choice Pitfall Olfactometer.* A two-choice pitfall olfactometer (Pierce et al., 1981) was used to bioassay volatile stimuli versus a solvent control. Two 8-ml glass vials were suspended from holes cut in the bottom of a 15-cm plastic petri dish. A filter paper disk treated with a pentane solution of the test stimulus was placed in one vial, and a disk treated with the same volume of pure pentane was placed in the other. Six replicates of 15 insects were employed for each stimulus. Bioassays were run for 2 hr in darkness at 30°C and ≈60% relative humidity in a controlled-environment chamber. At the end of the 2-hr period, the numbers of insects in the control and stimulus vials were recorded.

The following general guidelines assured maximal responsiveness. Test insects were 4- to 10-week-old adults of mixed age and sex from low density cultures (< 1500 insects/kg diet). They were sifted from cultures, aerated in darkness for ≈48 hr in a 2-liter flask with clean, moist air, and counted into holding vials ≈2 hr before a bioassay. After a bioassay, insects were allowed to feed at least 4 days before being reused.

### *Bioassay Experiments*

Twelve experiments were performed with *C. pusillus*. The first four used pentane extracts of Porapak Q-trapped beetle volatiles or fractions thereof. The next five assayed the attractiveness of synthetic I–III. Two experiments then

compared synthetic and beetle-produced pheromones. The final experiment tested the attractiveness of synthetic I plus (±)-II to segregated male and female insects. When bioassays were conducted to test isolated compounds versus their synthetic counterparts, the solutions of natural material were calibrated by gas chromatography (GC) against solutions of synthetic material of known concentration to ensure that the same absolute amounts of a pheromone were bioassayed. Pitfall olfactometer data were analyzed using the Student's $t$ test. Arena olfactometer data were analyzed with the Neuman-Keuls test modified for testing proportions (R. D. Routledge,[6] personal communication).

*Chemical and Instrumental Methods*

Hewlett-Packard 5830A and 5880A capillary gas–liquid chromatographs fitted with flame-ionization detectors were used for analytical gas chromatography. Capillary columns coated with SP-1000 (30 m × 0.66 mm ID, glass), Superox-4 (48 m × 0.5 mm ID, glass), or Carbowax 20 M (30 m × 0.32 mm ID, fused silica) were used, with temperature programing from 50 to 200°C. Injector temperature and detector temperatures were 260° and 275°C, respectively. For preparative GC, a Varian 1200 chromatograph fitted with a 10:1 effluent splitter, flame-ionization detector, and a thermal gradient collector (Brownlee and Silverstein, 1968) was used. The stainless steel column (3 m × 3.2 mm OD) was packed with 10% SP-1000 on Supelcoport (100/120 mesh). Temperature programing from 70° to 200°C was used in preparative separations with injector and detector at 240° and 275°C, respectively. Helium was used as carrier gas for all instruments.

In a typical preparative separation, the 10-ml solution of insect volatiles was cooled and concentrated to 50 μl with a stream of nitrogen in a conical-bottomed vial so that the residue could be removed easily by syringe. The concentrate was injected onto the column, and fractions were collected in 20 cm × 1.6 mm OD glass tubes (Brownlee and Silverstein, 1968). The tubes were capped with rubber Cryocaps for short-term storage or sealed for long-term storage at −30°C. The purity of collected samples was monitored by capillary GC.

Low-resolution mass spectra were obtained with a Hewlett-Packard 5985B GC-MS-DS system with electron impact ionization at 70 eV. The transfer line was maintained at 240°C, and the ion source at 200°C. Capillary columns used were fused silica (DB-1, 15 m × 0.25 mm ID, and SP-1000, 30 m × 0.32 mm ID). Coupled GC–high resolution mass spectra were taken on a Varian MAT 212 GC/HRMS, using an SS 200 data system. A 25-m × 0.21-mm ID Carbowax 20M fused silica column was used, with temperature programing from 50° to 200°C at 5°C/min.

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[$^1$H] NMR spectra were recorded on a Bruker WM 400 MHz instrument, in either 1.2 mm or 5 mm ID NMR tubes, using $CDCl_3$ (99.8%-D, Merck, Sharpe, and Dohme) or $D_6$-benzene (99.96%-D, Merck, Sharpe, and Dohme) as solvents. FTIR spectra were taken in the gas phase on a Nicolet 60SX FTIR coupled to a Hewlett-Packard 5792A capillary gas chromatograph.

Microhydrogenation of crude volatiles was done in a thick-walled 2-ml vial with a tight-fitting rubber septum. Pentane (250 $\mu$l), crude extract (50 $\mu$l), and a few grains of 10% Pd on carbon were stirred under 10 psi of $H_2$ for 1 hr.

Dodecanolide was synthesized by Baeyer-Villiger oxidation of dodecanone (Kaiser and Lamparsky, 1978).

## RESULTS

*Beetle and Frass Volatiles.* The initial stages of isolation and identification of volatiles from *C. pusillus* aerated with no food provided extracts containing only beetle-produced compounds. The rate of production of volatiles varied considerably between aerations, possible due to variations in the age of the beetles. Figure 1 shows a chromatogram of the Porapak Q-trapped volatiles from *C. pusillus* (mixed sex and age). Arena olfactometer bioassays of the extract confirmed Quaife's (1980) conclusion that there was a definite positive response to beetle volatiles (Table 1, Exp. 1). The response at 27 bh indicated the presence of a potent pheromone, and a high response was still seen at a concentration three orders of magnitude greater.

Four fractions (preparative GC) of the crude volatiles (Figure 1, Exp. 2), consisting of: forerun, all compounds eluting before compound I; compound I; impure compound II; and afterrun, all compounds eluting after compound II;

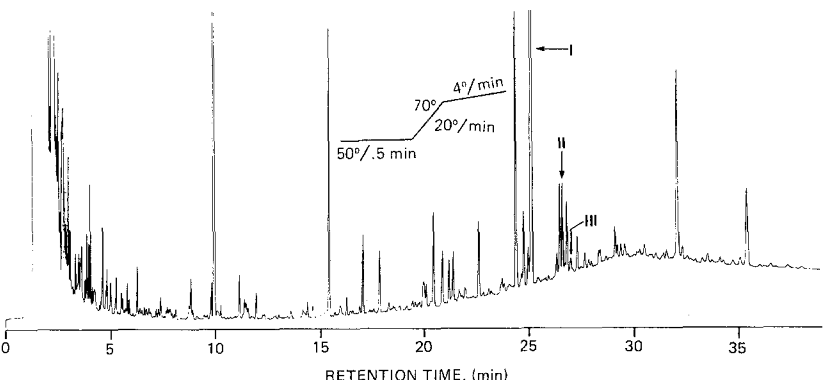


FIG. 1. Gas–liquid chromatogram of the pentane extract of Porapak Q-trapped *C. pusillus* volatiles (SP-1000 capillary column). Numbererd peaks represent macrolides I–III.

TABLE 1. RESPONSE OF *C. pusillus* OF MIXED SEX AND AGE IN ARENA OLFACTOMETER BIOASSAYS[a]

| Exp. | Stimulus | Dose[b] | Response (%)[c] |
|---|---|---|---|
| 1 | Pentane | 25 μl | 11.0a |
| | Beetle volatiles | 27 bh | 43.0b |
| | | 300 | 78.0c |
| | | 3,000 | 92.0c |
| | | 13,200 | 87.0c |
| | | 26,400 | 93.0c |
| 3 | Pentane | 20 μl | 11.0a |
| | Beetle volatiles | 3,400 bh | 92.0c |
| | I | 3,400 bh | 81.0c |
| | II | 3,400 bh | 46.0b |
| | Afterrun | 3,400 bh | 39.0b |
| 4 | Pentane | 20 μl | 4.0a |
| | I | 1,500 bh | 32.0cd |
| | I + forerun | 1,500 bh | 18.0bc |
| | I + II | 1,500 bh | 44.0d |
| | I + afterrun | 1,500 bh | 20.0bc |
| 5 | Synthetic I | 2 μg | 22.0a |
| | Synthetic I + (±)-II | 2 μg + 2.3 μg | 44.0b |
| | Synthetic I + (±)-II | 2 μg + 4.7 μg | 53.0b |
| | Synthetic I + (±)-II | 2 μg + 9.4 μg | 51.0b |
| 7 | Pentane | 20 μl | 6.0a |
| | Synthetic I | 4.0 μg | 44.0b |
| | Synthetic I + (±)-II | 4.0 μg + 200 ng | 69.0c |
| | Synthetic I + (±)-II | 4.0 μg + 400 ng | 54.0b |
| | Synthetic I + (±)-II | 4.0 μg + 800 ng | 52.0b |
| | Synthetic I + (±)-II | 4.0 μg + 1.6 μg | 52.0b |
| | Synthetic I + (±)-II | 4.0 μg + 4 μg | 46.0b |
| | Synthetic I + (±)-II | 4.0 μg + 8 μg | 49.0b |

[a]Ninety insects tested per stimulus.
[b]bh = beetle-hour, μl = microliter, μg = microgram, ng = nanogram.
[c]Percentages followed by the same letter are not significantly different, Neuman-Kuels test modified for testing proportions ($P < 0.05$).

were bioassayed in the pitfall olfactometer (Figure 2, Exp. 2). Only I exhibited attraction comparable to the crude extract, while the forerun appeared to be repellent. When the possibly attractive fractions were bioassayed in the arena olfactometer (Table 1, Exp. 3), I was confirmed to be highly attractive and again was comparable to the unfractionated extract. The afterrun and II were also attractive compared to the solvent control. Thus, most of the activity in the beetle volatiles was due to I, the major component. Combinations of I with the other fractions were tested at low stimulus doses so that any enhancement of response to I could be clearly seen. The combination of I and II produced the

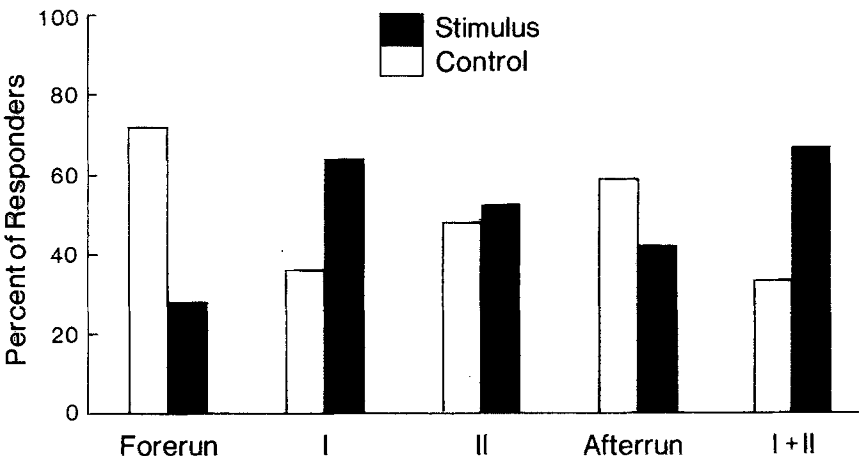


FIG. 2. Response in Exp. 2 of *C. pusillus* to preparative GC fractions of *C. pusillus* volatiles in the two-choice pitfall olfactometer.

highest response, which was not, however, significantly higher than to I alone (Table 1, Exp. 4).

Comparision of chromatograms of frass volatiles with beetle volatiles (Figures 1 and 3) showed the same compounds, I and II, were present. When insects were aerated on a food source (rolled oats), the production of I and II increased by at least an order of magnitude so that they comprised >60% of the total volatiles (Figure 4).

Examination by GC-MS of volatiles from sexually segregated beetles disclosed that males produced large amounts of I and II, while only a trace of I could be detected in the females' extract. The amount of I in the females' extract

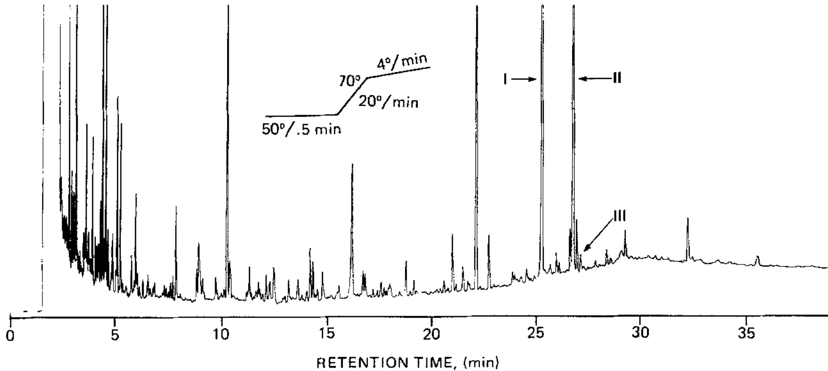


FIG. 3. Gas-liquid chromatogram of Porapak Q-trapped *C. pusillus* frass volatiles (SP-1000 capillary column). Numbered peaks represent macrolides I–III.

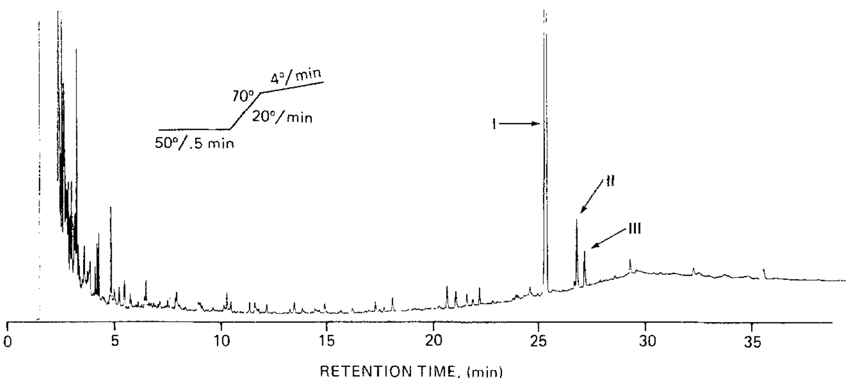


FIG. 4. Gas-liquid chromatogram of Porapak Q-trapped volatiles from *C. pusillus* feeding on oats (SP-1000 capillary column). Numbered peaks represent macrolides I–III.

was so small that it could have been due to a few incorrectly sexed insects or to traces of I adsorbed onto the females' exoskeletons.

At this point, compounds I and II were isolated, identified, and synthesized. Compound I was isolated from approximately $9 \times 10^6$ bh of *C. pusillus* volatiles in >97% purity. Compound II was isolated from approximately $8.4 \times 10^4$ gh of frass volatiles in >95% purity.

*Spectroscopic Identification of Pheromones.* Examination of the mass spectrum of I showed a fragmentation pattern (Figure 5) very similar to that of (*Z*)-3-dodecen-11-olide, a pheromone of *C. ferrugineus* (Wong et al., 1983), suggesting I was a structural isomer of that compound. The highest observed peak in the mass spectrum was *m/z* 196, indicating a possible molecular formula of $C_{12}H_{20}O_2$ and three sites of unsaturation. This formula was later confirmed by GC-HRMS (calc. for $C_{12}H_{20}O_2$: 196.1458; obs.: 196.060). GC-FTIR showed a nonconjugated carbonyl at 1735 $cm^{-1}$. Microscale hydrogenation showed the presence of one reducible olefin bond. Thus, the olefin and the carbonyl are

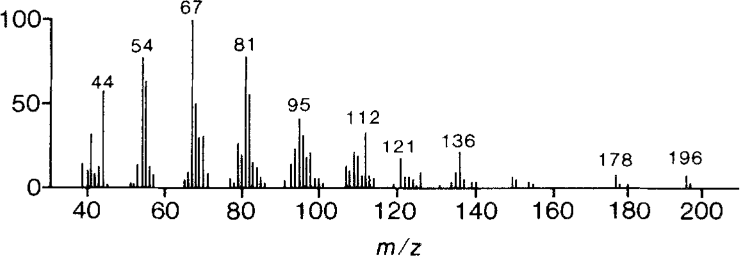


FIG. 5. Unit resolution mass spectrum of I.

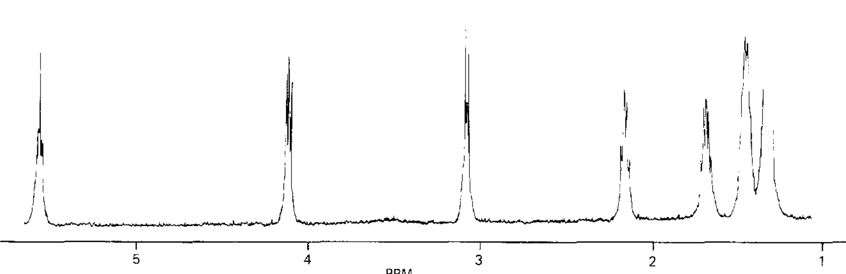


FIG. 6. [$^1$H] NMR (400 MHz) of I in $CDCl_3$.

responsible for two sites of unsaturation. A closer examination of the mass spectrum showed the first significant fragment to be at *m/z* 178 ($M^+ - 18$), corresponding to loss of $H_2O$. Significantly, there was no peak at $M^+ - 15$, possibly indicating that there were no methyl groups. In the *m/z* 40–140 range there were clusters of peaks separated by 14 mass units, indicative of a hydrocarbon chain.

Analysis of the [$^1$H] NMR spectrum (Figure 6) of I in $CDCl_3$ disclosed a multiplet at $\delta$ 5.55 corresponding to two coupled, overlapping hydrogens with $J = 10$ Hz, consistent with hydrogens on a *Z* double bond. A two-proton triplet ($J = 5.25$ Hz) at $\delta$ 4.09 was assigned to a methylene group adjacent to an ether or ester oxygen and was coupled to another methylene group. A doublet ($J = 6.8$ Hz) with additional fine splitting, corresponding to two hydrogens, at $\delta$ 3.05, was coupled to the olefin hydrogens. The simplicity of the splitting pattern and the chemical shift of this signal suggested it was due to an isolated methylene group between the double bond and a carbonyl. A two-hydrogen multiplet at $\delta$ 2.12, which collapsed to a triplet ($J = 6.6$ Hz) when decoupled from the olefin hydrogens, was identified as a second allylic methylene group. Another two-hydrogen multiplet at $\delta$ 1.65 was coupled to the methylene at $\delta$ 4.09, with its chemical shift suggesting it was due to hydrogen $\beta$ to an oxygen. There were 10 additional methylene protons ($\delta$ 1.25–1.51) and no other high-field resonances, i.e., no methyl groups, suggesting a cyclic structure to account for the remaining site of unsaturation. In addition, the structure was probably one large ring with no branch points as there were no methine or methyl hydrogens.

The macrolide structure I was proposed on the basis of the evidence above. The structure of the carbon skeleton was proved by hydrogenation of I to dodecanolide, which gave a mass spectrum and GC retention times on several columns identical to synthetic dodecanolide. Final confirmaiton of the structure was obtained by synthesis (Miller et al., 1983).

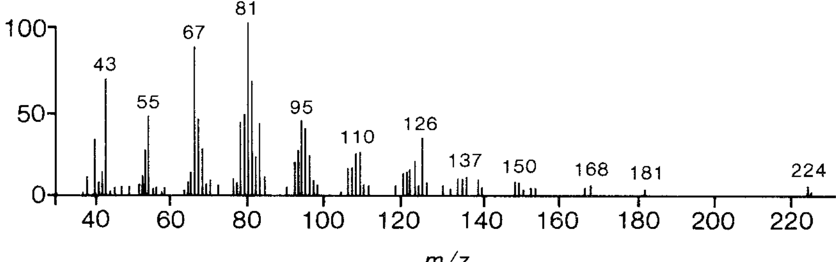


FIG. 7. Unit resolution mass spectrum of II.

The mass spectrum of II (Figure 7) showed similarities to that of I. The highest observed peak in the mass spectrum was at *m/z* 224, suggesting a molecular formula of $C_{14}H_{24}O_2$, which was later confirmed by GC-HRMS (calc. for $C_{14}H_{24}O_2$: 224.1776; obs.: 224.140). This formula requires three sites of unsaturation. Closer examination of the mass spectrum revealed small but distinct peaks at *m/z* 209 ($M^+ - 15$, loss of $CH_3$) and at *m/z* 206 ($M^+ - 18$, loss of $H_2O$). In addition, the spectrum in the region *m/z* 40–140 exhibited clusters of peaks separated by approximately 14 mass units, indicative of a hydrocarbon chain in the molecule. Coupled GC-FTIR of II indicated a nonconjugated carbonyl (1735 $cm^{-1}$), accounting for one site of unsaturation. Catalytic hydrogenation of II, followed by GC-MS analysis of the hydrogenated products, revealed one carbon–carbon double bond, thus accounting for a second site of unsaturation. A cyclic structure was postulated to account for the third.

The [$^1$H] NMR spectrum ($CDCl_3$) of II isolated from *C. pusillus* frass volatiles (Figure 8) showed two coupled single-hydrogen resonances at $\delta$ 5.32 and 5.38 ($J$ = 10.5 Hz), indicating a *Z* disubstituted double bond. A one-hydrogen multiplet at $\delta$ 4.98 was coupled ($J$ = 6.1 Hz) to a three-hydrogen methyl doublet at $\delta$ 1.22, suggesting a methine hydrogen on a carbon $\alpha$ to an oxygen, as in an ester. Two coupled ($J$ = 15 Hz) multiplets at $\delta$ 2.43 and $\delta$ 2.22 were assigned as a methylene group $\alpha$ to a carbonyl. Irradiation of one of these signals (at $\delta$ 2.43) resulted in the partial collapse of a one-hydrogen multiplet at $\delta$ 1.83, locating one hydrogen $\beta$ to the carbonyl. Irradiation of this signal resulted in the partial collapse of two one-hydrogen multiplets at $\delta$ 2.32 and $\delta$ 1.93. The chemical shifts of these signals suggested they were due to allylic hydrogens. This was confirmed by irradiation of the olefinic hydrogen at $\delta$ 5.32, which resulted in partial collapse of the multiplets at $\delta$ 2.32 and $\delta$ 1.93. Thus, the double bond was confirmed to be $\delta$, $\epsilon$ to the carbonyl.

Two additional allylic hydrogens were located by irradiation of the signal due to the other olefinic hydrogen ($\delta$ 5.38), which partially collapsed two one-hydrogen multiplets at $\delta \approx 2.20$ and $\delta$ 1.73. The remaining 11 hydrogen resonances (together with the three-hydrogen methyl doublet) formed an overlapped multiplet at $\delta$ 1.10–1.65, characteristic of hydrocarbon chains. Thus, the struc-

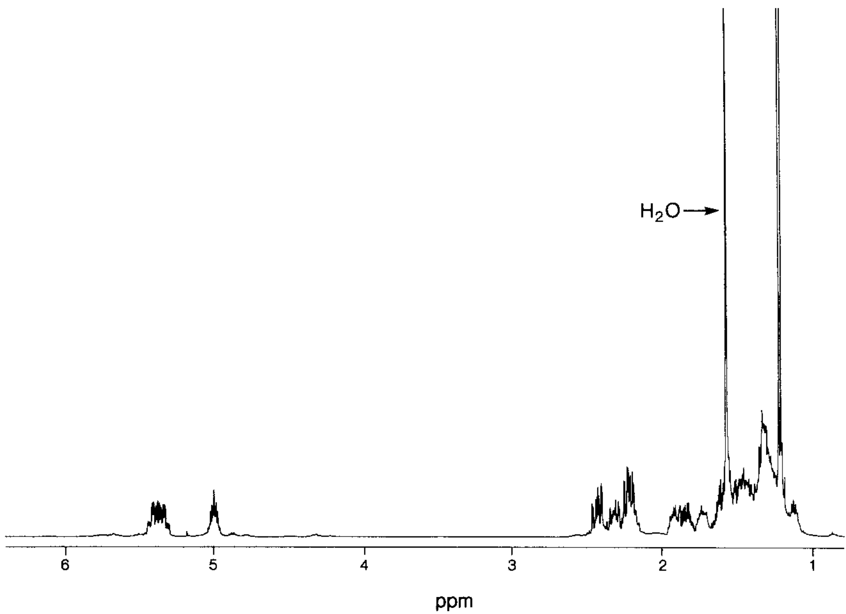


FIG. 8. [$^1$H] NMR (400 MHz) of II in $CDCl_3$.

ture of II was postulated to be a 14-membered lactone ring with a methyl group on the carbon $\alpha$ to the oxygen of the ester function, and a *Z* double bond four carbons removed from the ester carbonyl, i.e., (Z)-5-tetradecen-13-olide (II). The carbon skeleton was confirmed by catalytic hydrogenation, which gave a compound of molecular weight 226 with a mass spectrum identical to that of tetradecan-13-olide (Kaiser and Lamparsky, 1978). Final confirmation of the structure of II was determined by synthesis (Millar et al., 1983).

In addition to I and II, a small amount of another macrolide (III) was detected in volatiles obtained from beetles with or without food. This compound was identified by comparison of GC and mass spectral data (Figure 9) to those of III isolated and tentatively identified from *Oryzaephilus* species (Pierce et al, 1984). Final confirmation of the structure was obtained by synthesis (Millar et al., 1983).

*Bioassays with Synthetic Pheromones.* Bioassays were carried out with synthetic compounds I, II, and III (Tables 1 and 2). Experiments with mixtures of I and ($\pm$)-II showed a small but significant synergism of I by ($\pm$)-II (Table 1, Exp. 5). A dose–response experiment for the attractive mixture of I:($\pm$)-II (1:1) in the arena olfactometer determined that the threshold concentration for significant activity was between 1 and 4 ng for each of I and ($\pm$)-II (Figure 10). The response then increased steadily to reach a maximum between 4 and 20 $\mu$g. At 20 $\mu$g, the response decreased significantly ($t$ test, $P < 0.01$) from that at 4

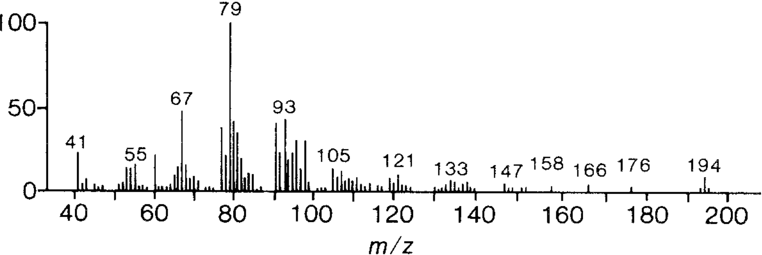


FIG. 9. Unit resolution mass spectrum of III.

μg, suggesting that sensory adaptation (Seabrook, 1977), disorientation due to atmospheric permeation (Nara et al., 1981), or arrestment downwind of the stimulus source (Wood et al., 1966) occurred.

A systematic test of the ratio of I and (±)-II in both types of bioassay disclosed that that the best ratio of I and (±)-II approached the naturally produced ratio of ≈20:1 (Table 1, Exp. 7; Table 2, Exp. 8). The responses with the two bioassay methods differed: in the enclosed chamber, pitfall olfactometer (Table 2, Exp. 8), but not in the arena olfactometer (Table 1, Exp. 7), the highest concentration of II was inhibitory. Despite numerous repetitions of bioassays with both methods, it was not possible to demonstrate that the response to I is synergized more by one enantiomer of II than the other, suggesting that the insects use either enantiomer equally well (Silverstein, 1979).

Although III was present only in trace amounts in volatile extracts, it was tested for attractiveness, as it has a very similar structure to I. Significant response to III was obtained at 1 and 10 μg, but not at 0.1 μg, indicating that the threshold for response was about 100× greater than for I (Table 2, Exp. 9). In addition, when III was tested as a concentration equal to that of a highly atractive unfractionated extract, there was no significant response (Table 2, Exp. 11). Tests for synergism of I and III revealed no consistent synergism, over a range of ratios of I:III of 100:1 through 10:1 (the natural ratio is ≈75:1).

Bioassays of synthetic compounds and preparative GLC fractions (a forerun; a fraction containing I, II, and III; an afterrun) of feeding *C. pusillus* (Table 3, Exp. 10) demonstrated three pertinent phenomena: (1) All the activity in the crude extract was recovered in the recombined fractions, indicating that no active compounds were destroyed by passage through the GC column. (2) The forerun (mainly food volatiles) induced a response comparable to that induced by the macrolides fraction, while the afterrun fraction was inactive. Combination of the forerun and macrolide fractions resulted in total recovery of activity. Thus, food volatiles are attractive, and they enhance the response to the pheromone fraction. (3) Synthetic I + (±)-II at the same concentrations as in the macrolide fraction exhibited activity comparable to the isolated macrolide fraction.

TABLE 2. RESPONSE OF *C. pusillus* OF MIXED AGE AND SEX IN PITFALL OLFACTOMETER BIOASSAYS[a]

| Exp. | Experimental stimulus | Amount of stimulus | Response ($\bar{X}$ ± SE)[b] Experimental stimulus | Pentane control |
|---|---|---|---|---|
| 8 | Synthetic I | 2.0 μg | 5.7 ± 1.4 | 1.2 ± 0.5* |
| | Synthetic I + (±)-II | 2.0 μg + 100 ng | 7.3 ± 1.1 | 1.5 ± 0.6* |
| | Synthetic I + (±)-II | 2.0 μg + 200 ng | 6.7 ± 1.7 | 2.0 ± 0.7* |
| | Synthetic I + (±)-II | 2.0 μg + 400 ng | 8.2 ± 1.2 | 2.2 ± 1.0* |
| | Synthetic I + (±)-II | 2.0 μg + 800 ng | 5.7 ± 0.4 | 1.3 ± 0.4* |
| | Synthetic I + (±)-II | 2.0 μg + 2 μg | 6.0 ± 0.6 | 3.3 ± 0.7* |
| | Synthetic I + (±)-II | 2.0 μg + 4 μg | 4.1 ± 0.8 | 1.3 ± 0.3* |
| | Synthetic I + (±)-II | 2.0 μg + 8 μg | 4.2 ± 0.9 | 3.0 ± 0.6 NS |
| 9 | Synthetic III | 10 ng | 7.2 ± 0.5 | 5.8 ± 0.8 NS |
| | Synthetic III | 100 ng | 6.0 ± 1.1 | 3.8 ± 1.1 NS |
| | Synthetic III | 1 μg | 7.0 ± 0.8 | 4.1 ± 1.2* |
| | Synthetic III | 10 μg | 9.5 ± 1.5 | 2.0 ± 0.4* |
| 10 | Volatiles of *C. pusillus* on oats | 660 bh | 8.8 ± 1.0 | 1.8 ± 0.6** |
| | Forerun fraction | 660 bh | 8.7 ± 1.4 | 2.8 ± 0.8* |
| | Macrolide fraction | 660 bh | 7.8 ± 1.1 | 3.8 ± 0.9* |
| | Aferrun fraction | 660 bh | 5.2 ± 0.8 | 4.8 ± 0.5 NS |
| | Forerun + macrolides | 660 bh | 11.5 ± 1.0 | 1.5 ± 0.6*** |
| | Recombined fractions | 660 bh | 9.0 ± 0.6 | 2.2 ± 0.5*** |
| | Synthetic I + (±)-II | 660 bh | 8.3 ± 1.5 | 2.3 ± 0.7* |
| 11 | Volatiles of *C. pusillus* on oats | 660 bh | 12.5 ± 0.5 | 1.8 ± 0.6*** |
| | Synthetic I | 660 bh[c] | 8.0 ± 0.6 | 4.7 ± 0.8** |
| | Synthetic I + (±)-II | 660 bh[c] | 8.5 ± 0.9 | 3.8 ± 0.8* |
| | Synthetic III | 660 bh[c] | 4.5 ± 1.0 | 4.8 ± 0.5 NS |
| | Macrolide fraction | 660 bh | 7.5 ± 0.7 | 2.5 ± 0.7** |

[a] N = 6 replicates, 15 insects/replicate.
[b] Significant response (*t* test) to experimental stimulus indicated by: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS = not significant.
[c] Concentrations of synthetic I, (±)-II, and III were calibrated relative to the crude extract of *C. pusillus* on oats. In absolute terms, this represented 850 ng of I, 43 ng of (±)-II, and 11 ng of III.

The latter finding was corroborated in another experiment (Table 2, Exp. 11), in which the isolated macrolides, synthetic I, and synthetic I + (±)-II all had similar levels of activity. The unfractionated extract was the most attractive stimulus, probably due to the presence of macrolides plus food volatiles. In this instance, there was no detectable synergism of I by (±)-II.

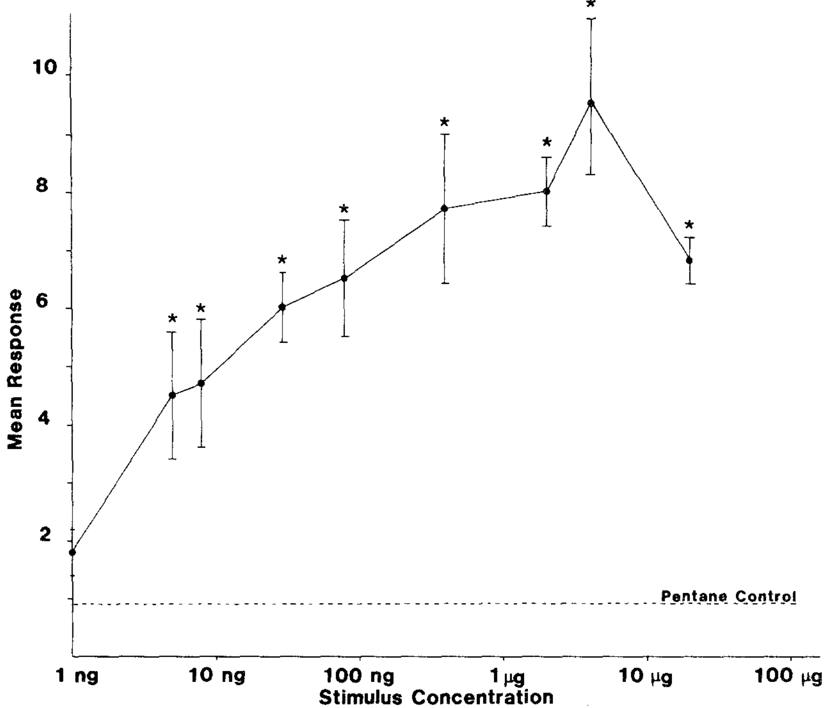


FIG. 10. Dose–response curve of *C. pusillus* to mixtures of I + (±)-II (1:1.2) in the arena olfactometer (Exp. 6). $N = 6$ replicates, 15 insects/replicate. Horizontal axis is in logarithmic scale. A significant response to a stimulus versus a solvent control is indicated by *, $P < 0.01$.

TABLE 3. RESPONSE OF MALE AND FEMALE *C. pusillus* OF MIXED AGE IN EXP. 12 TO MIXTURE OF I AND (±)-II IN PITFALL OLFACTOMETER BIOASSAYS[a]

| Sex of test insects | Experimental stimulus | Amount of stimulus | Response ($\bar{X}$ ± SE)[b] Experimental stimulus | Pentane control |
|---|---|---|---|---|
| Males | I + (±)-II | 400 ng + 40 ng | 4.8 ± 0.7 | 0.8 ± 0.3** |
| | I + (±)-II | 2 µg + 200 ng | 6.2 ± 0.6 | 0.7 ± 0.3*** |
| | I + (±)-II | 10 µg + 1 µg | 7.0 ± 0.6 | 0.8 ± 0.3*** |
| Females | I + (±)-II | 400 ng + 40 ng | 2.5 ± 0.7 | 1.0 ± 0.4 NS |
| | I + (±)-II | 2 µg + 200 ng | 5.2 ± 0.9 | 2.0 ± 0.3* |
| | I + (±)-II | 10 µg + 1 µg | 6.5 ± 0.7 | 1.8 ± 0.4** |

[a] $N = 6$ replicates, 15 insects/replicate.
[b] Significant response ($t$ test) to experimental stimulus indicated by: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS = not significant.

Both male and female beetles responded to several concentrations of a 10 : 1 mixture of I : (±)-II (Table 3, Exp. 12), indicating that I and II are true aggregation pheromones. Males responded at a lower stimulus concentration than females and had a consistently higher level of overall response.

## DISCUSSION

In preliminary bioassays of crude extracts, the overall response obtained was rather low, but the differences in response to stimuli and solvent controls were highly significant. As experimentation proceeded, it was found that the best responses to volatile stimuli were obtained from insects 4–10 weeks old, raised in low-density cultures (< 1500 insects/kg diet) (Pierce et al., 1983), and starved for 48 hr in darkness. In addition, if insect-produced volatiles were removed by flushing air through the flask in which the insects were being starved, the responses improved considerably.

Arena olfactometer bioassays confirmed Quaife's (1980) conclusion that there was a definite positive response to beetle volatiles (Table 1, Exp. 1). The response at 27 bh is comparable to the threshold sensitivity for *C. ferrugineus* (Borden et al., 1979; Wong et al., 1983), and indicates a potent pheromone in *C. pusillus*. A very high response was still obtained at a stimulus concentration three orders of magnitude greater than threshold.

Comparison of the chromatograms of volatiles from starved beetles, beetles on food, and frass showed that compounds I–III were present in all three extracts. It is noteworthy that the production of I–III increased dramatically when the insects were aerated on food, as evidenced by the phermone components being the only major peaks in that extract (Figure 4). This result supports the hypothesis that I, II, and III are aggregation pheromones released to attract other members of the species to a suitable food source.

Aeration of sexually segregated insects showed that only males produced the attractants I and II, while III was not found in either sex. This confirmed Quaife's (1980) observation that only the extracts of male volatiles were attractive to insects of either sex. Borden et al. (1979) had also observed that the aggregation pheromones of the closely related *C. ferrugineus* were male-produced.

Bioassays with synthetic compounds I and II gave a more detailed picture of the pheromone system. Experiments with mixtures of I and (±)-II showed significant synergism of I by (±)-II. This result also indicates that if the insects produce one enantiomer of II preferentially, their response is not inhibited by the presence of the antipode. In addition, numerous attempts to demonstrate synergism by one enantiomer of II showed no preference for either enantiomer, indicating that the insects may, in fact, use either enantiomer equally well. Thus, species specificity of response could not be maintained by repellency of *C. pus-*

*illus* by either enantiomer of II produced by another species. The dose–response curve of a mixture of I and (±)-II indicated that there was a maximum concentration threshold, beyond which the response decreased, as was found for *C. ferrugineus* (Borden et al., 1979) and two *Ozyzaephilus* species (Pierce et al., 1981).

The positive response obtained to large doses of III suggests several possibilities. First, the synthesis of III required the reduction of a diyne precursor, overreduction of which would give I. Thus, traces of I in the synthetic III may have been responsible for the response obtained. Alternatively, the structural similarity of III to I may result in its acting as an analog of I at high concentrations. In view of the fact that the threshold response dose of III is $\approx$ 100 times greater than that of the most active component, I, it is suggested that III is not an aggregation pheromone.

The fact that food volatiles, alone or in mixtures with the pheromone components, were attractive is not surprising considering that the test insects had been starved for 48 hr before the bioassay. It remains to be seen whether food volatiles will compete in effectiveness with pheromones in the field, when the insects have not been starved.

The higher response by males than females to a mixture of I and (±)-II was rather curious, as the compounds are male-produced, and the opposite sex is usually preferentially attracted to aggregation pheromones in cases where the pheromones are produced by one sex (Borden, 1983). However, when the responses of both sexes to the pheromone plus host volatiles is fully elaborated, the differences between sexes may be slight.

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# AGGREGATION PHEROMONES OF THE GRAIN BEETLE, *Cryptolestes turcicus* (COLEOPTERA: CUCUJIDAE)[1]

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**Abstract**—Two biologically active macrolides were isolated from Porapak Q-captured beetle and frass volatiles of *Cryptolestes turcicus* (Grouvelle) and identified spectroscopically as (*Z,Z*)-5,8-tetradecadien-13-olide (I) and (*Z*)-5-tetradecen-13-olide (II). Natural I was active alone and was synergized by inactive II. The pheromones were male-produced but attractive to both sexes. Pheromone production increased dramatically when insects were aerated on a food source. Pure (*R*)- and (*S*)-I were inactive, but mixtures of (*R*)- and (*S*)-I were active, the first reported instance of enantiomeric synergism in the Cucujidae.

**Key Words**—*Cryptolestes turcicus*, Coleoptera, Cucujidae, aggregation pheromone, macrolide, (*Z,Z*)-5,8-tetradecadien-13-olide, (*Z*)-5-tetradecen-13-olide, enantiomeric synergism.

## INTRODUCTION

The grain beetle, *Cryptolestes turcicus* (Grouvelle), is a worldwide pest of stored products (Howe and Lefkovitch, 1957) and a secondary feeder, infesting mainly broken or milled grain. As aggregation pheromones are produced by *C. ferrugineus* (Stephens) (Wong et al., 1983) and *C. pusillus* (Schönherr) (Millar et al., 1985), we hypothesized that *C. turcicus* also utilizes aggregation pheromones. The macrolides I and II have been isolated from the Porapak Q-trapped

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STRUCTURES I and II.

beetle and frass volatiles and identified as pheromones for this species. The syntheses of the attractive compounds I and II are reported elsewhere (Millar et al., 1983; Millar and Oehlschlager, 1984).

## METHODS AND MATERIALS

All experimental methods (insect rearing, capture and extraction of insect and frass volatiles, isolation and identification of active compounds, and bioassay procedures) were as described for *C. pusillus* (Millar et al., 1985).

Ten bioassay experiments were performed. The first tested the attractiveness of clean food, beetle-infested food, live beetles, and frass, respectively, in an arena olfactometer to beetles of mixed sex and age, the next four tested the attractiveness of various pentane extracts of beetle volatiles and fractions thereof, in the arena and pitfall olfactometers, four more tested (±)- and/or enantiomeric I and II, and mixtures thereof, and the final experiment tested the response of segregated male and female insects to (±)-I.

## RESULTS

*Bioassays.* The first experimental bioassay was designed to ascertain whether or not *C. turcicus* employed aggregation pheromones (Table 1, Exp. 1). There was no response to noninfested food volatiles. There was a definite attraction to the odor of infested media, live beetles, and frass, evidently due to attractive compounds produced by the beetles. A pentane extract of Porapak Q-captured beetle volatiles (Figure 1) was attractive to *C. turcicus* over a range of doses in both the arena and pitfall olfactometers (Table 1, Exp. 2; Table 2, Exp. 3). The minimum dose required to elicit a significant response was between 500 and 1000 beetle-hours (1 bh = the volatiles produced by one beetle in one hour), approximately an order of magnitude higher than for *C. pusillus* (Millar et al., 1985) or *C. ferrugineus* (Wong et al., 1983).

TABLE 1. RESPONSE OF *C. turcicus* OF MIXED AGE AND SEX IN ARENA OLFACTOMETER BIOASSAYS[a]

| Exp. No. | Stimulus | Amount of stimulus | (%) Response[b] |
|---|---|---|---|
| 1 | Pure air | | 6.0a |
| | Mixed oats and cracked wheat (≈1:1) | 13 g | 6.0a |
| | Used culture media, oats and cracked wheat | 13 g | 37.0b |
| | Live beetles, mixed sex and age (≈12000 insects) | 4.1 g | 23.0b |
| | Beetle frass | 5.6 g | 37.0b |
| 2 | Pentane | 20 μL | 5.0a |
| | Porapak Q-trapped beetle volatiles | 2,000 bh | 24.0b |
| | | 5,000 bh | 28.0bc |
| | | 10,000 bh | 41.0c |
| | | 20,000 bh | 46.0c |
| 4 | Pentane | 20 μL | 9.0a |
| | Fraction 1 | ≈20,000 bh | 5.0a |
| | 2 | ≈20,000 bh | 4.0a |
| | 3 | ≈20,000 bh | 7.0a |
| | 4 (compound II) | ≈20,000 bh | 8.0a |
| | 5 (compound I) | ≈20,000 bh | 35.0b |
| | 6 | ≈20,000 bh | 5.0a |
| | 7 | ≈20,000 bh | 7.0a |

[a]Ninety insects tested per stimulus.
[b]Percentages followed by the same letter are not significantly different, Neuman-Kuels test modified for testing proportions ($P < 0.05$).

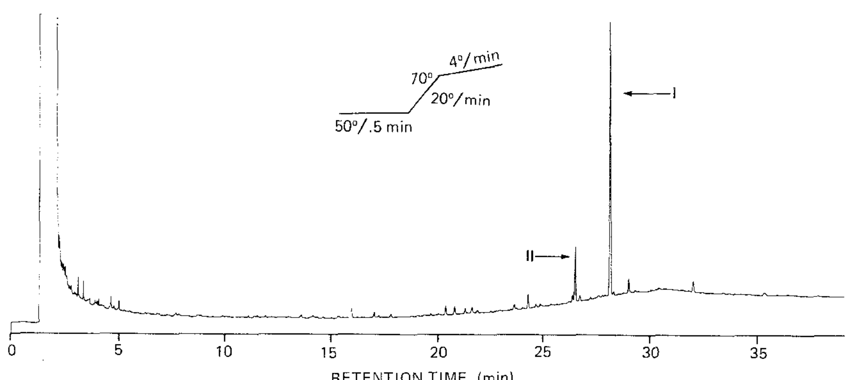


FIG. 1. Gas–liquid chromatogram of Porapak Q-trapped *C. turcicus* volatiles (SP-1000 capillary column). Numbered peaks represent macrolides I and II.

TABLE 2. RESPONSE OF *C. turcicus* OF MIXED AGE AND SEX IN PITFALL OLFACTOMETER BIOASSAYS[a]

| Exp. No. | Experimental stimulus | Amount of stimulus | Response ($\bar{X}$ ± SE)[b] Experimental stimulus | Solvent control |
|---|---|---|---|---|
| 3 | Pentane extract of *C. turcicus* volatiles | 500 bh[c] | 6.3 ± 1.0 | 8.0 ± 1.1 NS |
| | | 1000 bh[c] | 9.5 ± 1.2 | 3.8 ± 0.9 * |
| | | 2000 bh[c] | 10.0 ± 0.5 | 4.5 ± 0.6 ** |
| 5 | *C. turcicus* frass volatiles | 3150 gh[c] | 7.2 ± 1.7 | 4.7 ± 1.8 NS |
| | | 315 gh[c] | 10.2 ± 0.7 | 1.8 ± 0.5 *** |
| | | 31.5 gh[c] | 10.5 ± 0.6 | 1.3 ± 0.2 **** |
| | On oats extract | 6200 bh | 9.7 ± 0.6 | 1.7 ± 0.8 *** |
| | | 620 bh | 9.3 ± 0.6 | 3.0 ± 0.4 *** |
| | | 62 bh | 5.7 ± 1.3 | 4.0 ± 1.2 NS |
| 6 | Synthetic (±)-I | 240 ng | 5.3 ± 0.6 | 5.5 ± 0.6 NS |
| | | 2.4 μg | 6.7 ± 0.8 | 4.0 ± 0.6 * |
| | | 24 μg | 9.8 ± 0.9 | 2.5 ± 0.6 ** |
| | Synthetic (±)-II | 200 ng | 4.2 ± 0.5 | 3.2 ± 0.8 NS |
| | | 2.0 μg | 5.3 ± 1.6 | 3.5 ± 1.0 NS |
| | | 20 μg | 3.3 ± 0.7 | 4.2 ± 0.9 NS |
| 7 | (*R*)-I | 5 μg[d] | 4.5 ± 1.5 | 1.5 ± 0.4 NS |
| | (*R*)-I : (*S*)-I, 95:5 | 5 μg[d] | 9.7 ± 1.2 | 1.8 ± 0.7 ** |
| | 90:10 | 5 μg[d] | 6.7 ± 1.0 | 2.0 ± 0.4 ** |
| | 80:20 | 5 μg[d] | 4.5 ± 1.1 | 0.3 ± 0.2 ** |
| | 60:40 | 5 μg[d] | 3.8 ± 1.2 | 0.8 ± 0.5 * |
| | 40:60 | 5 μg[d] | 8.2 ± 1.3 | 1.3 ± 0.4 ** |
| | 20:80 | 5 μg[d] | 8.0 ± 0.8 | 3.0 ± 0.7 ** |
| | 10:90 | 5 μg[d] | 5.0 ± 1.0 | 2.0 ± 0.8 * |
| | 5:95 | 5 μg[d] | 6.7 ± 1.1 | 3.7 ± 1.4 NS |
| | (*S*)-I | 5 μg[d] | 4.8 ± 0.7 | 2.8 ± 1.2 NS |
| 8 | Synthetic (±)-I | 10 μg | 4.5 ± 0.8 | 1.0 ± 0.7 ** |
| | (±)-I + (±)-II | 10 μg + 400 ng | 7.7 ± 1.3 | 1.8 ± 0.3 ** |
| | | 10 μg + 800 ng | 7.3 ± 1.5 | 0.8 ± 0.7 ** |
| | | 10 μg + 1.67 μg | 7.0 ± 1.3 | 2.7 ± 0.7 ** |
| | | 10 μg + 3.33 μg | 10.3 ± 0.8 | 1.8 ± 0.9 ** |
| | | 10 μg + 6.67 μg | 10.5 ± 1.6 | 0.83 ± 0.4 ** |
| | | 10 μg + 10 μg | 8.3 ± 0.8 | 1.7 ± 0.5 *** |
| | | 10 μg + 20 μg | 8.7 ± 0.6 | 2.1 ± 0.7 *** |

TABLE 2. Continued

| Exp. No. | Experimental stimulus | Amount of stimulus | Response ($\bar{X}$ ± SE)[b] Experimental stimulus | Solvent control |
|---|---|---|---|---|
| 9 | (±)-I | 5 μg | 7.3 ± 1.3 | 3.0 ± 0.4 ** |
| | (±)-I + (R)-II | 5 μg + 1.67 μg | 7.1 ± 1.6 | 0.7 ± 0.2 ** |
| | (±)-I + (S)-II | 5 μg + 1.67 μg | 8.3 ± 1.3 | 2.0 ± 0.4 ** |
| | (±)-I + (R)-II | 5 μg + 3.33 μg | 8.5 ± 1.5 | 1.0 ± 0.4 ** |
| | (±)-I + (S)-II | 5 μg + 3.33 μg | 4.5 ± 0.9 | 2.2 ± 0.4 * |
| | (±)-I | 1 μg | 5.0 ± 1.2 | 4.8 ± 0.7 NS |
| | (±)-I + (R)-II | 1 μg + 330 ng | 3.5 ± 0.7 | 2.7 ± 0.9 NS |
| | (±)-I + (S)-II | 1 μg + 330 ng | 6.7 ± 1.5 | 2.7 ± 0.8 NS |
| | (±)-I + (R)-II | 1 μg + 670 ng | 7.5 ± 1.4 | 2.5 ± 0.6 * |
| | (±)-I + (S)-II | 1 μg + 670 ng | 4.7 ± 1.4 | 3.5 ± 0.4 NS |

[a]N = 6 replicates, 15 insects/replicate.
[b]Significant response (t test) to experimental stimulus indicated by: ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS = not significant.
[c]bh = beetle-hours, gh = gram-hours of frass volatiles where 1 gh = 1 g frass aerated for one hour.
[d]5 μg of (R)-I + (S)-I.

Pitfall bioassays showed that pentane extracts of Porapak Q-captured frass volatiles were highly attractive at low amounts (Table 2, Exp. 5). At high doses, the response was not significantly different than that to the pentane control stimulus, suggesting sensory adaptation, disorientation, or arrestment. Analysis by GC-MS of frass volatiles revealed that I and II were major components (Figure 2), so it is reasonable that the frass and insect volatiles should exhibit comparable biological activity.

A pentane extract of Porapak Q-captured volatiles of *C. turcicus* feeding on oats also elicited a strong positive response (Table 2, Exp. 5), with the threshold for response being between 62 and 620 bh. GC-MS analysis (Figure 3) showed that the major components of the extract were again I and II. The lower response threshold to volatiles from feeding as compared to starved insects may reflect the larger absolute amounts of volatiles per bh produced by feeding beetles.

Bioassays of seven preparative GC fractions of the crude beetle volatiles (10% SP-1000 on Supelcoport, 3 m × 3.2 mm OD stainless-steel column, programed 70–200°C at 4°C/min) revealed that only fraction 5, consisting primarily of I, was active by itself (Table 1, Exp. 4). Fraction 4, consisting of II, was inactive.

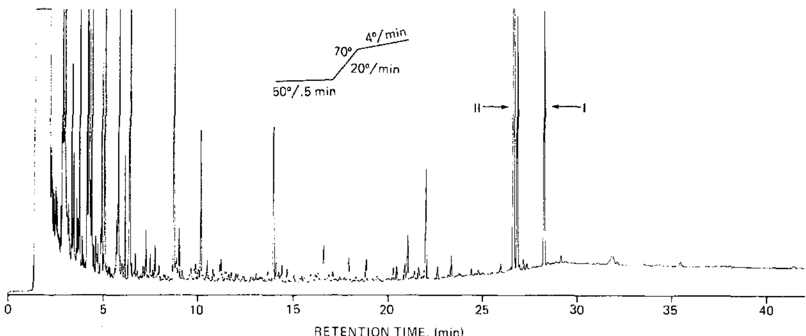


FIG. 2. Gas–liquid chromatrogram of Porapak Q-trapped *C. turcicus* frass volatiles (SP-1000 capillary column). Numbered peaks represent macrolides I and II.

*Spectroscopic Identification of I and II.* Compounds I and II were isolated by preparative GC (as described above) from $4.3 \times 10^6$ bh of beetle volatiles. Analysis by capillary GC (Millar et al., 1985) showed the isolates to be >96% and >95% pure, respectively. The highest observed mass in the mass spectrum of I was at *m/z* 222 (Figure 4), and a typical macrolide-type fragmentation pattern, along with the retention time, suggested that I was similar to macrolides isolated from *C. pusillus* (Millar et al., 1985). Microscale hydrogenation gave a compound with *m/z* 226, with an identical mass spectrum and GC retention time to the macrolide obtained on catalytic hydrogenation of (*Z*)-5-tetradecen-13-olide, i.e., tetradecan-13-olide (Millar et al., 1985). This analysis confirmed the carbon skeleton of I was a 14-membered lactone with a methyl group attached to the carbon $\alpha$ to the ester oxygen. Thus, it was necessary only to identify the placement of the olefin or acetylene bonds.

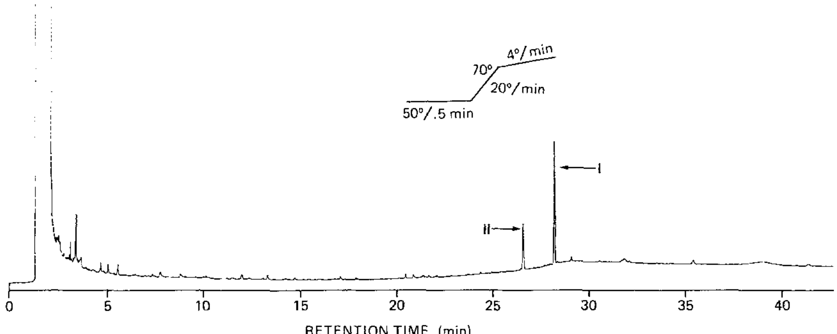


FIG. 3. Gas–liquid chromatogram of Porapak Q-trapped volatiles from *C. turcicus* feeding on oats (SP-1000 capillary column). Numbered peaks represent macrolides I and II.

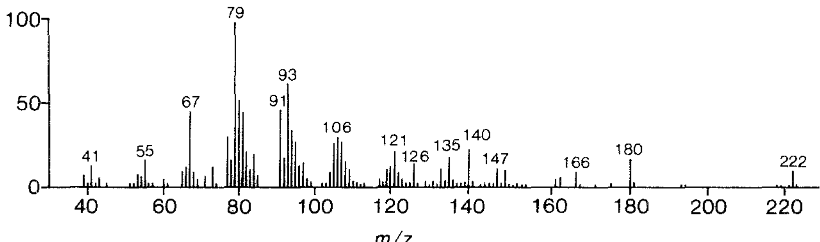


FIG. 4. Unit resolution mass spectrum of I.

The 400-MHz [$^1$H] NMR spectra ($CDCl_3$) of a sample of I isolated from *C. turcicus* volatiles by preparative GC (Figure 5) revealed signals due to four olefinic hydrogens, one at $\delta$ 5.27 and three that overlapped at $\delta$ 5.40. The isolated proton at $\delta$ 5.27 was coupled ($J$ = 9.75 Hz) to one of the other vinyl hydrogen signals, confirming at least one $Z$ double bond. There was a single-hydrogen multiplet at $\delta$ 5.03, coupled to a methyl group at $\delta$ 1.24 ($J$ = 6.1 Hz), corresponding to the methine hydrogen of C-13. A one-hydrogen doublet of triplets at $\delta$ 3.15, geminally coupled ($J$ = 15.1 Hz) to a hydrogen in a three-hydrogen multiplet at $\delta$ 2.20–2.43 and also coupled to the olefin multiplet ($J \simeq$ 9.75 Hz) at $\delta$ 5.40, was identified by the coupling and downfield shift as a bisallylic hydrogen. Thus, there was a skipped diene system. The other bisallylic hydrogen

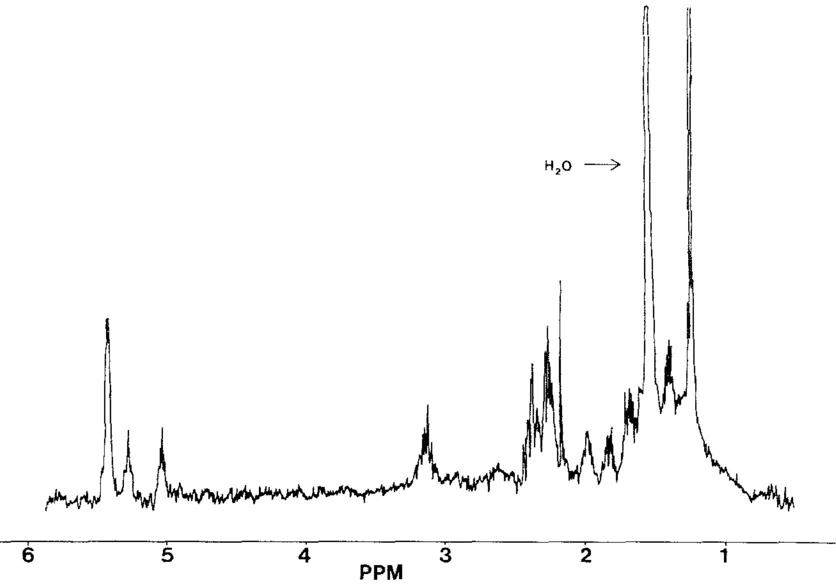


FIG. 5. [$^1$H] NMR (400 MHz) of I in $CDCl_3$.

gave the geminal coupling in the δ 2.20–2.43 multiplet. The other two hydrogens in that multiplet were identified as allylic hydrogens on different carbons by partial collapse of their signals upon irradiation of the olefin multiplets. The two remaining allylic hydrogens were located at the same time, at δ 1.98, and as a part of a four-hydrogen multiplet at δ 1.56–1.77.

The two coupled hydrogens ($J$ = 14.5 Hz) of a methylene α to the carbonyl (δ 2.27 and 2.40, respectively) were identified by their chemical shift and splitting patterns. Irradiation of the signal at δ 2.40 resulted in partial collapse of a one-hydrogen multiplet at δ 1.83 and of part of the four-hydrogen multiplet at δ 1.56–1.77, indicating a methylene group β to the carbonyl. Finally, irradiation of the one-hydrogen signal at δ 1.83 resulted in partial collapse of signals due to two previously identified allylic hydrogens at δ 1.98 and in the multiplet at δ 2.20–2.43, thus locating the first double bond between C-5 and C-6. In addition, as irradiation of the single olefinic hydrogen signal at δ 5.27 removed a large coupling (≈9.75 Hz) from the allylic hydrogen at δ 1.48, the δ 5.27 hydrogen must be located on C-5 and previous analysis had shown this hydrogen to be on a $Z$ disubstituted double bond. The other double bond must be between C-8 and C-9, as the analysis above had identified a single methylene between the two double bonds. The remaining four unassigned hydrogens, on C-11 and C-12, were assigned to the remainder of the four-hydrogen multiplet at δ 1.56–1.77 and to the two proton multiplet at δ 1.40.

Thus, the only unknown factor was the geometry of the double bond between C-8 and C-9, the protons of which were completely overlapped. Changing the solvent from $CDCl_3$ to $C_6D_6$ gave no improvement. Shift reagents were not tried, as the recovered sample had to be used uncontaminated for bioassays. Final confirmation that the C-8, -9 bond geometry was $Z$ was obtained by synthesis (Millar and Oehlschlager, 1984).

GC and GC-MS analyses of *C. turcicus* volatiles (Figure 1) showed a compound with the same retention times on several columns and the same mass spectrum as macrolide II isolated from *C. pusillus* volatiles (Millar et at., 1985). In addition, a sample of II isolated by preparative GC gave an identical NMR spectrum to II isolated from *C. pusillus*, proving conclusively that the compounds had the same structure. However, the two species may produce different enantiomers or a different ratio of the enantiomers of II.

*Bioassays with Synthetic I and II.* The racemic and enantiomeric forms of I and II were synthesized (Millar et al., 1983; Millar and Oehlschlager, 1984). When synthetic racemic I and II were bioassayed individually, II was inactive by itself (Table 2, Exp. 6), corroborating the data on naturally produced II (Table 1, Exp. 4). The threshold for significant response for racemic I was between 240 ng and 2.4 μg (Table 2, Exp. 6), considerably higher than for *C. pusillus* to individually active pheromones (Millar et al., 1985). Bioassay of a series of solutions ranging from pure ($R$)- to pure ($S$)-I showed that *C. turcicus* did not

respond to pure (*R*)- or (*S*)-I at the 5-$\mu$g level (Table 2, Exp. 7). However, there were significant levels of activity at all but one of the enantiomeric ratios tested (5:95, *R*:*S*), indicating a synergism between enantiomers. This is the first evidence of enantiomeric synergism outside of the Scolytidae, in which it occurs in *Gnathotrichus sulcatus* (Borden et al., 1976) and *Ips pini* (Lanier et al., 1980). The minimal attractiveness of the pure enantiomers of I was confirmed on a separate occasion, when pitfall bioassays of either enantiomer over a range of amounts (10 ng–10 $\mu$g) produced no significant response.

A test for synergism between racemic I and II over a range of ratios (25:1 to 1:2) (Table 2, Exp. 8) showed that all mixtures used were attractive, and that ratios of 3:1 and 3:2 of I : II were significantly more attractive than I alone (Neuman-Kuels test, $P < 0.05$), indicating that II does synergize I when used in approximately the natural ratio. The fact that all the stimuli tested were highly attractive suggested that the range of ratios exhibiting significant synergism could probably be extended by testing at lower concentrations. However, this hypothesis was not confirmed in bioassays of combinations of racemic I with the enantiomers of II, using two ratios of I:II at two concentrations (Table 2, Exp. 9). When 5 $\mu$g of I was used, all mixtures of I with the enantiomers of II were equally attractive (Neuman-Kuels test, $P > 0.05$). When the concentration of I was reduced to 1 $\mu$g, only the mixture containing 670 ng of (*R*)-II showed any significant synergism. Thus, it is suggested that (*R*)-II, or a mixture of enantiomers of II synergizes I.

In order to determine whether pheromones were produced by one sex, sexually segregated insects were aerated, and the Porapak Q-captured volatiles were analyzed by GC-MS. There was no trace of I or II in females' volatiles. The extract of males' volatiles, however, contained small but definite amounts of I and II, thus indicating that the pheromones are exclusively male-produced. In a final experiment, racemic I was bioassayed with sexually segregated beetles (Table 3, Exp 10). Both sexes responded, although the response of males was not significant at the 1-$\mu$g level. The test did show that I was attractive to both sexes and is thus a true aggregation pheromone.

## DISCUSSION

During the initial stages of isolation and identification of volatiles, adult beetles were aerated with no food. This procedure provided volatiles wherein only beetle-produced compounds were present. It was noted that the rate of production of volatiles, as evidenced by the absolute amounts of I and II produced in different extracts of approximately the same number of beetle-hours of aeration, varied considerably. This may have been due to variations in the age of the beetles, as in related species (Pierce et al., 1984). When the insects were aerated on oats, the rate of pheromone production increased by approxi-

TABLE 3. RESPONSE OF MALE AND FEMALE *C. turcicus* OF MIXED AGE IN EXP. 10 TO SYNTHETIC (±)-I IN PITFALL OLFACTOMETER BIOASSAYS[a]

| | | | Response ($\bar{X}$ ± SE)[b] | |
|---|---|---|---|---|
| Sex | Experimental stimulus | Amount of stimulus | Experimental stimulus | Pentane control |
| Male | (±)-I | 10 μg | 3.7 ± 0.5 | 1.0 ± 0.4 ** |
| | (±)-I | 2.5 μg | 3.0 ± 0.7 | 0.2 ± 0.2 ** |
| | (±)-I | 1 μg | 1.7 ± 0.4 | 1.3 ± 0.4 NS |
| Female | (±)-I | 10 μg | 4.3 ± 0.7 | 1.2 ± 0.6 * |
| | (±)-I | 1 μg | 2.2 ± 0.3 | 0.8 ± 0.2 * |

[a]N = 6 replicates, 10 insects/replicate.
[b]Significant response (*t* test) to experimental stimulus indicated by: **, $P < 0.01$; *, $P < 0.05$; NS = not significant.

mately an order of magnitude, supporting the hypothesis that I and II are aggregation pheromones released to attract other members of the species to a scarce food supply. It also suggests that the insects need a food source to produce pheromones optimally (Burkholder, 1982).

As for *C. pusillus* (Millar et al., 1984) and other similar species (Pierce et al., 1983; Mikolajczak et al., 1983), the insects had to be "conditioned" before reasonably consistent and reproducible response in the bioassay could be obtained. The best responses were obtained from insects approximately 6–10 weeks old, raised in a low-density culture (< 1500 insects/kg diet), and starved for 48 hr in the darkness. If insect-produced volatiles were removed by flushing air through the flask in which the insect were being starved, the response was improved.

The aggregation pheromones were determined to be exclusively male-produced, as has been found in other long-lived coleopteran stored-product pests (Burkholder, 1982). Compound I is mainly responsible for aggregation behavior, and a mixture of enantiomers of I must be present to elicit aggregation behavior. Compound II apparently synergizes I, with the *R* enantiomer being primarily responsible for synergistic effects. However, the optimally attractive mixture of I and II could be quite complex, as it could in effect be regarded as a four-component mixture, where a specific ratio of each of the enantiomers of I and II would be necessary to elicit the maximum response. Thus, the various mixtures tested so far could be considerably different than the optimum ratio, which would account for the relatively high dosages of pheromones to produce a good response, in comparison to *C. ferrugineus* (Wong et al., 1983) or *C. pusillus* (Millar et al., 1985). To date, it has not been possible to determine the enan-

tiomeric composition of naturally produced I and II from *C. turcicus* by use of chiral NMR shift reagents or by use of a GC column coated with chiral stationary phase. Future bioassays with the enantiomers of I and II may reveal more details of this complex question.

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# DEFENSIVE SECRETION PRODUCTION IN THE TENEBRIONID BEETLE, *Zophobas atratus*
## Effects of Age, Sex, and Milking Frequency

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**Abstract**—Beetles were milked of their abdominal defensive secretion at three different frequencies over the first 36 days of adult life. Secretion production decreased greatly with age from a maximum of 94 nmol/day at 4–6 days to a basal level of about 25 nmol/day at 30–40 days. Ethyl- and methylquinone comprised the bulk of the secretion and were most strongly affected by age. Benzoquinone was most strongly affected by milking frequency. An average adult produced an estimated 4445 nmol of total secretion in its 5-month life-span but had reservoirs large enough to store 11,000 nmol. Males produced more secretion than females because of their larger size and inherent sexual differences. As they aged, mated females showed a more rapid decrease in production that virgin females. The defensive system appears to be programed to charge glands rapidly with secretion early in adult life and fall of to a low recharge rate little affected by discharge.

**Key Words**—Chemical defense, *Zophobas atratus*, Coleoptera, Tenebrionidae, defensive secretion, gland recharge, defensive quinone, quinone production.

## INTRODUCTION

Two important characteristics of a chemical defensive system are how quickly it is initially charged with secretion and how quickly a depleted system can be recharged with additional secretion. Although much work has been done on arthropod chemical defenses (for reviews see: Roth and Eisner, 1962; Eisner and Meinwald, 1966; Jacobson, 1966; Schildknecht, 1971; Meinwald et al. 1978; Rockstein, 1978; Blum, 1981), little is known about these two characteristics.

Eisner (1958) reported high recharge rates for the carabid beetle, *Brachinus*



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*ballistarius* LeConte, and Tschinkel (1975) found most tenebrionid species "milked" to apparent depletion can release some secretion again in a day or two, but either of these may be the result of physical effects such as the secretion of water into the defensive reservoirs rather than de novo synthesis (Tschinkel, 1975). The dytiscid beetle, *Agabus obtusatus*, recharges its prothoracic glands in three weeks, while *A. seriatus* requires four weeks to replace 61% of a prothoracic component (Fescemeyer and Mumma, 1983). Carrel (1984) found adult millipedes recharge glands at a constant rate for 100 days, implying a depleted individual needs four months to reload its glands.

Recent studies indicate that age, sex, and size may influence defensive secretion production in beetles. Newly eclosed adults have little secretion, while older adults have much more (Kaneshisa, 1978; Dettner and Schwinger, 1982; Classen and Dettner, 1983). Secretion composition and titer have also been found to vary with age and season (Miller and Mumma, 1974; Dettner, 1979; Newhart and Mumma, 1979a,b; Dettner and Schwinger, 1982; Classen and Dettner, 1983), and some of this variation is likely due to variation in population structure (Dettner, 1979; Classen and Dettner, 1983). Gland size and sex may also have important effects (Kaneshisa, 1978; Dettner and Schwinger, 1982).

In this study, we examined the effect of adult age, sex, and discharge frequency on the defensive secretion production of the abdominal glands in the tenebrionid beetle, *Zophobas atratus*.

## MATERIALS AND METHODS

*Zophobas atratus* is a large tenebrionid beetle from Zamorano, Honduras, laboratory-reared since 1978. Like *Z. rugipes*, it has two pairs of defensive glands. The prothoracic pair exudes the phenolic (phenol, *m*-cresol, and *m*-ethylphenol) secretion, and the abdominal pair, a quinonic mixture (1,4-benzoquinone, 2-methyl-1,4-benzoquinone, and 2-ethyl-1,4-benzoquinone; hereafter referred to as benzoquinone, methylquinone, and ethylquinone, respectively) in equilibrium with an aqueous phase (Tschinkel, 1969). Because the prothoracic glands are difficult to milk, they were not examined in this study.

*Zophobas atratus* was selected for this study because (1) it is easily reared in the laboratory, (2) the abdominal defensive glands are everted so all secretion can be collected in each milking, and (3) the defensive secretion is simple (see above).

Experimental adults were individually maintained at 30°C in plastic boxes with screened tops and provided bran, cricket feed, and water ad libitum.

*Collection and Analysis of Secretion.* Secretion was collected by roughly handling a beetle until its glands were everted and then wiping these free of secretion with a small piece of filter paper. The paper was immediately extracted in carbon disulfide and stored at −20°C.

Samples were analyzed within a few days of collection as a single injection into a Varian Aerograph 1400 gas chromatograph with a FID detector (carrier gas: nitrogen, 30 ml/min; column, $\frac{1}{8}$ in. × 5 ft aluminum, 10% OV-101 on HP Chromosorb W (AW DMSW, 80–100 mesh), oven: 150°C). The peak areas were converted to mass by a Shimadzu C-RIA Chromopac recording data processor calibrated with external standards, and the mass was converted to nanomoles for analysis of variance (ANOVA).

*Egg Production.* Pairs of beetles were maintained in screen-topped plastic boxes at 30°C. The bottom of each plastic box contained a 2-cm layer of Wondra flour covered with a wire screen. Females laid eggs through the screen into the flour. On top of the screen, beetles were fed a chunky baked mixture (unsuitable for oviposition) of bran, cricket feed, Wondra flour, and water. Using a No. 40 U.S. Standard Testing sieve, eggs were sifted from the flour every seven days and counted.

Data were log transformed to normalize and analyzed by analysis of variance (ANOVA) and Duncan's multiple-range test.

## RESULTS

*Experiment I: Effect of Age, Sex, and Milking Frequency.* Beetles were sexed and weighed as pupae and assigned to one of three treatment groups: (1) milked every three days; (2) milked every six days; or (3) milked every 12 days. Each group contained 15 males and 15 females, which all eclosed within 24 hr. Pupal weights were used to eliminate any effects of body size; average pupal weight of males was 0.774 ± 0.089 g and females 0.687 ± 0.073 g. Statistical comparisons were made on the total nanomoles of secretion per gram pupal weight collected at the end of three consecutive 12-day periods. For example, at 12 days, we compared the sum of the first four milkings of the three-day group, the first two milkings of the six-day group, and the first of the 12-day group. Separate analyses were performed for total quinone content and each component quinone.

Age of adult beetles had a tremendous negative effect on their defensive secretion production (Figure 1). Between day 12 and 36, total quinone production fell 64% from 1410 nmol to 509 nmol quinone/g pupal weight (ANOVA, $P < 0.0001$), while methyl- and ethylquinone decreased 72% and 68%, respectively ($P < 0.0001$, Figure 1). Age accounted for 53% of the total variance of these three measures or 80% of the explained variance. No other factor or interaction accounted for more than 4% of the total variation in these measures.

Production of benzoquinone, on the other hand, decreased only 28% over the same period ($P < 0.001$, Figure 1) with age accounting for 7% of the total variance. As a result of the differential decrease in production of each quinone,

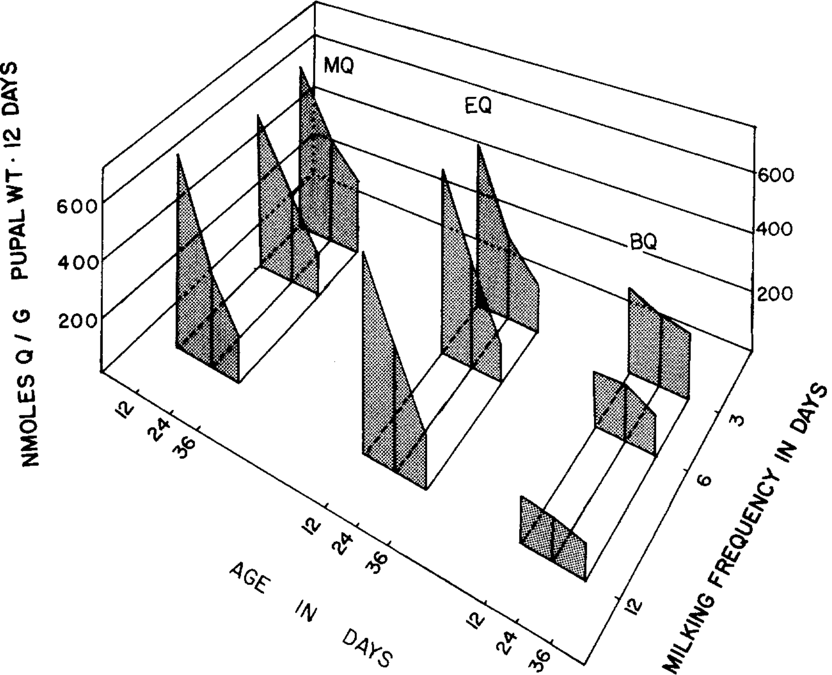


FIG. 1. Production of individual quinones in relation to age and milking frequency (in days). From day 12 to day 36, MQ decreased 72%, EQ, 68%, and BQ, 28%. More frequent milking stimulated production of MQ and BQ. For both MQ and BQ, production decreased more slowly in the three-day group than in the six- or 12-day groups. MQ = methylquinone, EQ = ethylquinone, BQ = benzoquinone, Q = quinone, PUPAL WT = pupal weight.

the composition of the newly produced secretion changed from an initial ratio of 3:3:1 to a final ratio of 1:1:1 of methyl-, ethyl-, and benzoquinone.

Milking stimulated benzoquinone production, causing a two-fold increase of the most frequently milked group over the least ($P < 0.05$; Figure 1). Milking frequency accounted for 22% of total benzoquinone variance. More frequent milking stimulated a small but significant ($P < 0.001$) increase in total and methylquinone (Figure 1) production and accounted for 3% and 4%, respectively, of their total variance.

Males not only produced 16% more total quinone per gram pupal weight than did females, but more of each quinone as well: 16% more methylquinone, 3% more ethylquinone, and 48% more benzoquinone. Total quinone and alkylquinone (methyl- and ethyl-) production was initially greater in females but decreased more rapidly than it did in males ($P < 0.001$, Figure 2). Although

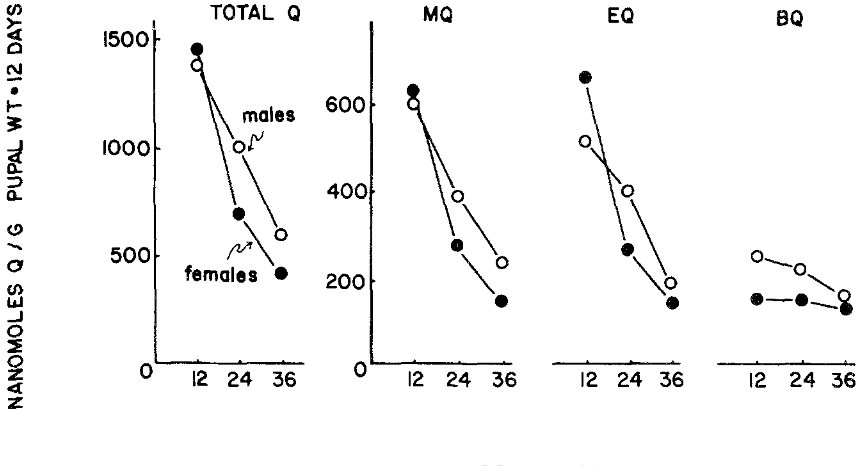


FIG. 2. Interaction of age with sex for total quinones and each component quinone. Males produced more of each measure than females. Production of total Q, MQ, and EQ decreased more quickly in females than in males. TOTAL Q = total quinones, MQ = methylquinone, EQ = ethylquinone, BQ = benzoquinone.

male benzoquinone production decreased 36% during the 36-day period, it always remained higher than female benzoquinone production. Sex accounted for 13% of the total variation in benzoquinone.

It is interesting that methyl- and ethylquinone showed nearly identical responses to all experimental factors (Figures 1 and 2). Duffy and Blum (1976) (in Blum, 1981) established that these alkylquinones are biosynthesized by a pathway independent of that for benzoquinone in *Zophobas rugipes*. If the total amount of quinones reflects defensive potency, then males are 13% more defensively potent per gram pupal weight as shown in Figure 2 or 31% more potent per beetle than females.

Cumulative secretion approached an upper limit with increasing age, indicating that biosynthesis decreased to some basal level (Figure 3). Alkylquinone and total quinone production decreased similarly, while benzoquinone production, which was not strongly affected by age, continued at nearly the same rate.

The average daily production per beetle (including data from experiment II) plotted against age (Figure 4), reveals a rapid decrease from a maximum rate of 94 nmol/beetle/day at 4–6 days to a basal level of about 25 at 30–40 days.

Thus, we found in the order of importance of effect: (1) age was the most significant factor, having its greatest effect on total and alkylquinone production; total quinone production was greatest at 4–6 days and decreased to a basal level at 54 days; (2) males produced more secretion than females because of and in-

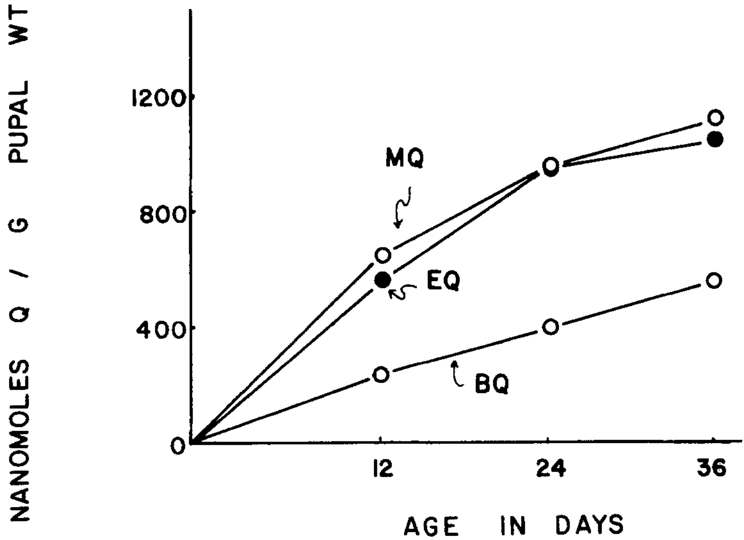


FIG. 3. Cumulative production of each quinone versus beetle age. This shows the approximate gland content in the absence of periodic milking. MQ = methylquinone, EQ = ethylquinone, BQ = benzoquinone, Q = quinone.

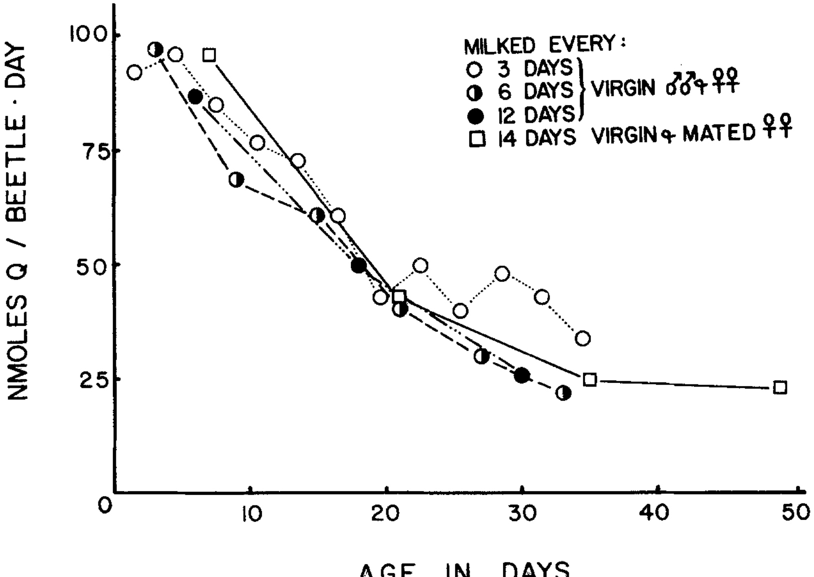


FIG. 4. Average daily secretion production per beetle. Each point is the average for its collection period and is plotted in the center of that period.

dependent of size differences; (3) milking frequency primarily stimulated benzoquinone production; and (4) the alkylquinones occurred in nearly equal quantities, comprised the bulk of the secretion, and were more strongly and almost equally affected by treatment factors.

*Experiment II: Effect of Egg Production.* Because the decrease in production between day 12 and day 24 (Figure 3) coincides with the onset of egg production in females, we tested the hypothesis that egg production had a negative effect on secretion production (perhaps by causing material to be shunted away from the defensive system to the reproductive system). This experiment differed from experiment I as follows: (1) beetles were placed into one of two treatment groups: virgin (isolated females) or mated (one male and one female with ad libitum, but unknown mating); (2) eggs were collected every seven days; (3) females were milked every 14 days; (4) the males were never milked; and (5) the experiment lasted 56 days. Quinone data were again converted to nanomoles, pupal weight-adjusted, log transformed, and analyzed by ANOVA.

As in experiment I, age accounted for the largest portion of the total variance of each measure: total quinones, 69%; methylquinone, 64%; ethylquinone, 69%; and benzoquinone, 17%.

Although mated females laid nearly twice as many eggs as virgin females ($P < 0.05$), mating did not significantly affect secretion production but did cause total and individual quinone production to decline more rapidly with age. This interaction explained 2% of the total variance of each measure (age by treatment interaction; $P < 0.05$). Egg production, however, was not significantly related to secretion production, perhaps because the beetle does not channel very much energy into defensive secretion production. A larger trade-off between secretion and egg production would be likely in species investing more energy in secretion.

These results suggest that the virgin females in the previous experiment were also reasonably representative of mated females with respect to secretion production.

## DISCUSSION

The rapid decline in quinone production suggests that the defensive system in *Z. atratus* is programed to charge glands rapidly early in adult life and drop off to a low recharge rate. Kaneshisa (1978) found defensive titers of newly eclosed tenebrionid beetles reached a maximum about one month after emergence, which agrees with our study. Carrel (1984) found millipedes recharge at a low constant rate for 100 days. Since this millipede passes its secretion from one molt to the next (Carrel, 1984), a high recharge rate immediately following a molt would be unnecessary and might explain the observed recharge rate. The high recharge rates in dytiscid beetles reported by Fescemeyer and Mumma

(1983) may reflect a different defensive strategy, i.e., discharge and loss of secretion, and the greater recharge this demands.

The older a *Z. atratus* is when it loses its defensive secretion, the longer its defenses are reduced. For example, by 12 days an average beetle produces 1035 nmol of secretion. At 24 and 36 days a beetle would require an additional 42 and 50 days, respectively, to produce another 1035 nmol. If all secretion were lost after 42 days (25% of an adult's life-span), it would never be completely replaced. However, if secretion were allowed to accumulate, older beetles would have greater defensive stores, assuming quinones are stable for this period. Calculations of reservoir volume indicate that a beetle could store up to 11,000 nmol, but an average beetle, which lives five months in the lab, could only produce 4445 nmol of secretion. Thus the reservoirs are never more than $\frac{1}{3}$ full of secretion, which probably adheres as a film on the densely folded walls.

Beetles probably retain secretion for most of their lives (4–12 months in the lab) because: (1) they use their secretion very efficiently—no secretion is lost by spraying or exuding and the densely folded reservoir walls help retain the secretion—so it is very unlikely all secretion would be lost in an encounter with a predator; (2) beetles probably encounter predators at a much lower frequency than they were milked in this experiment; and (3) they also have a pair of thoracic defensive glands which exude a phenolic secretion as a supplementary defense. The thoracic secretion is only released when the applied stimulus is relatively severe, while the abdominal glands are probably the first line of defense in encounters with predators. Animals pinched and poked with forceps while standing, readily everted their abdominal glands but did not release their thoracic secretion. When lifted off the substrate, 43% released their thoracic secretion, while 100% everted their abdominal glands ($N$ = 23) (personal observation). The thoracic secretion not only flows over the beetle's cuticle and may present a greater threat to the beetle's own well-being, but it is more likely to be lost than the abdominal secretion.

The decrease in quinone production parallels decreasing reproductive potential with increasing age in insects. Older individuals have lower reproductive potential (Engelmann, 1970) and therefore expend less energy on survival, i.e., defensive secretion production.

Because production of alkylquinones is so much greater than that of benzoquinone, in the absence of reservoir depletion and secretion degradation, the quinone ratio changes very little with increasing age. An unusual quinone ratio could only occur if an individual lost most of its secretion after 36 days and was therefore recharging when production of the three quinones is nearly equal.

Males produced 31% more secretion per beetle than females. Perhaps males seek out females and thus are exposed to more predation, which in turn favors those which produce more secretion. This is likely the case in *Glomeris mar-*

*ginata*, where males produce almost 1.6 times as much defensive secretion per gram body weight as females (Carrel, 1984).

Finally, our study indicates that quantitative and qualitative studies of defensive secretion cannot be effectively investigated without considering the effects of age, sex, or previous discharge. We believe that the low recharge rate or basal rate is a reflection of this beetle's life history as well as the efficiency with which the secretion is used.

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# CONSTITUENTS OF OSMETERIAL SECRETION OF PRE-FINAL INSTAR LARVAE OF CITRUS SWALLOWTAIL, *Papilio demodocus* (ESPER) (LEPIDOPTERA: PAPILIONIDAE)[1]

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**Abstract**—The defensive osmeterial secretion of pre-final instar larvae of the citrus swallowtail, *Papilio demodocus* (Lepidoptera: Papilionidae) was found to contain methyl 3-hydroxybutanoate, 3-hydroxybutanoic acid, $\alpha$-pinene, myrcene, limonene, $\beta$-phellandrene, (*Z*)-ocimene, (*E*)-ocimene, $\beta$-caryophyllene, (*E*)-$\beta$-farnesene, and germacrene-A, as well as a further number of unidentified sesquiterpenoid constituents. The presence of germacrene-A in the secretion was inferred from the formation of $\beta$-elemene under certain GC conditions. Larvae of the second, third, and fourth instars produce qualitatively similar secretions. Remarkable quantitative differences were found between the secretions of individual larvae. These variations could not be correlated with the diet on which the larvae were fed, their sex, instar, or color form. However, in a number of larvae the two prongs of the osmeterium were found to produce quantitatively different secretions.

**Key Words**—*Papilio demodocus*, Lepidoptera, Papilionidae, defensive secretion, osmeterial secretion, monoterpenes, sesquiterpenes, capillary GC, injector programing, GC-MS, [$^{13}$C]NMR.

[1]Abstracted in part from the MSc thesis of Z. Munro, University of Stellenbosch, March 1981.



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## INTRODUCTION

Larvae of the butterfly family Papilionidae possess a defensive gland, the osmeterium, situated mid-dorsally behind the head. Normally, this gland is invisible but when the larva is irritated, the osmeterium is extruded as a prong- or fork-like structure, this being an evagination of the cervical membrane. The gland is apparently evaginated by blood pressure and withdrawn by special retractor muscles (Eisner and Meinwald, 1965). A defensive secretion is produced by the osmeterium and, in the everted position, the prongs of the organ appear damp or even glistening with the secretion.

The structure and ultrastructure of the osmeterium and the chemical nature of the osmeterial secretion of a considerable number of papilionids have been the subject of various papers (Eisner and Meinwald, 1965; Crossley and Waterhouse, 1969; Eisner et al., 1970; López and Quesnel, 1970; Seligman and Doy, 1972; Burger et al., 1978; Honda, 1980a, b, 1981, 1983b).

In several of the species investigated so far, the osmeterial secretion of the fifth instar was found to contain 2-methylpropanoic acid and 2-methylbutanoic acid as the major constituents. It has been found, however, that the last two instars of the papilionid *Battus polydamas* secrete the sesquiterpenes $\beta$-selinene and selin-11-en-4$\alpha$-ol (Eisner et al., 1971) while 3-hydroxybutanoic acid has been identified as a component of the penultimate larval instar of *Papilio aegeus* (Seligman and Doy, 1972). Honda (1980b) has found that the osmeterial secretions of the last as well as the penultimate instars of a number of papilionids in the genera *Luehdorfia* and *Atrophaneura* contain terpenoid and sesquiterpenoid constituents, whereas the last two instars of two *Graphium* species produce osmeterial secretions containing 2-methylpropanoic acid, 2-methylbutanoic acid, and their methyl and ethyl esters.

In a paper on the chemical nature of the osmeterial secretion of *P. demodocus* (Burger et al., 1978), we have reported 2-methylpropanoic acid, 2-methylbutanoic acid, and their methyl and ethyl esters as the major constituents of the defensive secretion of final instar larvae. These compounds were found to be totally absent from the osmeterial secretion of first- and second-instar larvae, which contained, instead, at least 15 mono- and sesquiterpenoids. More recently Honda (1980a) has identified a number of mono- and sesquiterpenoids in the osmeterial secretion of the third- and fourth-instar larvae of *P. protenor demetrius*. In a subsequent paper, the same author (Honda, 1981) reported the identification of a large number of terpenoids in the secretions of *P. machaon hippocrates*, *P. memnon thunbergii*, *P. helenus nicconicolens*, *P. bianor dehaanii*, and *P. maackii tutanus* and compared the quantitative ratios in which these terpenoids and methyl 3-hydroxybutanoate are present in the osmeterial secretions of the fourth-instar larvae. As far as the defensive potential of the osmeterial

secretion is concerned, Honda (1983a) has found the acids, esters, and some of the terpenoids to be toxic and/or repellent to two ant species.

We now wish to report the results of an investigation of the chemical composition of the defensive osmeterial secretion of pre-final instar larvae of *Papilio demodocus* Esper.

## METHODS AND MATERIALS

*Collection of Secretion.* Larvae were collected from citrus trees in the district of Stellenbosch during three consecutive summer seasons, between 1978 and 1980, and taken to the laboratory where their rearing was continued on *Citrus limon* (cultivar Eureka). For this purpose the larvae were kept on branches of the host plant supported in Erlenmeyer flasks filled with water. The larvae were maintained under a 16:8-hr light–dark cycle at a temperature of 22°C. The secretion (37 mg) from 400 larvae was collected in glass capillaries (25 × 1 mm ID) which had been sealed off at one end and slightly flared at the other. To collect the secretion, the larvae were held at an angle, either between the fingers or on a leaf, and the flared end of the capillary pressed lightly at the back of the head, whereon the larva normally extended the two prongs of the osmeterium into the capillary. The still-extended osmeterium was then slowly withdrawn from the capillary, leaving the secreted material as an opaque suspension on the capillary wall. As larvae produced progressively smaller quantities of material on repeated collection, secretion was only collected once or twice from individual larvae during each instar. Capillaries with the collected material were stored at −30°C in a vial with a Teflon-lined screw cap until needed for analytical experiments.

*Analytical and Synthetic Methods.* Gas chromatographic analyses (GC) were carried out with a Carlo Erba model 4160 gas chromatograph. Retention time comparisons were done with a Varian model 3700 gas chromatograph. Both gas chromatographs were equipped with flame ionization detectors and were interfaced to a Hewlett-Packard 3385A Automation System integrator.

Initially all comparative GC analyses were carried out on a Pyrex capillary column coated with Carbowax 20 M (40 m × 0.3 mm ID, film thickness 0.3 μm). For subsequent GC and gas chromatographic–mass spectrometric analyses (GC-MS), this column as well as three Pyrex capillary columns, coated with OV-73 (40 m × 0.3 mm ID, film thickness 1.0 μm), SE-30 (40 m × 0.3 mm ID, film thickness 3.0 μm) and Carbowax 1000 (15 m × 0.3 mm ID, film thickness 0.15 μm) were used. All the columns were deactivated and coated statically according to procedures developed by Grob (Grob et al., 1977; Grob, 1980). For GC analyses, samples were introduced into the injectors of the gas chromatographs in the normal manner, by splitless injection of a solution of the material

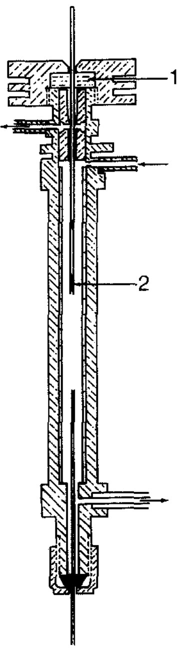



FIG. 1. Solventless injection of the osmeterial secretion of *Papilio demodocus*: (1) septum; (2) capillary sealed in the middle and containing the sample in the lower, open-ended section.

in dichloromethane, carbon disulfide, or deuterobenzene. For reliable quantitative determinations and to avoid the introduction of artifacts, samples were also introduced into the injector of the Carlo Erba gas chromatograph without using any solvent, by inserting a capillary, containing the sample, into the injector through a hole in the septum which was normally closed with an empty capillary (Figure 1). To facilitate quantitative transfer of the sample onto the column, the injector was operated in the splitless mode for ca. 2 min. This unorthodox technique resulted in pressure fluctuations in the injector as the pressure regulator did not always return to its original setting. Consequently, this technique was not used for retention-time determinations.

GC-MS analyses were carried out with a Varian MAT 311A mass spectrometer (IV: 70 eV) interfaced to a Varian Aerograph 2700 gas chromatograph by means of a restricting glass capillary. Mass spectrometric information was recorded on magnetic tape using a Varian SpectroSystem 100MS data handling system. The glass capillary columns described above were employed using analogous gas chromatographic conditions.

Preparative GC separation of components of the osmeterial secretion was carried out with a Perkin-Elmer model 900 gas chromatograph equipped with a

glass column (14 m × 3.0 mm ID), packed with 2.5% Carbowax 20 M on 60–80 mesh Chromosorb WAW-DMCS, flow rate 24 ml $H_2$/min, temperature programed at 1.5°C from 50°C to 205°C, injector 190°C, detector 210°C. An effluent splitter was used which allowed 5% of the effluent to flow to the flame ionization detector. Fractions were precipitated in stainless-steel needles (Luer, Inox No. 18, 250 × 0.8 mm ID) by employing alternate cold (−50°C) and hot (200°C) zones along the middle 70-mm section of the needles. The collected secretion (37 mg), diluted to 75 μl with dichloromethane (Merck, residue analysis grade) was injected in ca. 6-μl quantities for preparative gas chromatographic isolation of components. After every fourth separation, the individual components were rinsed from the needles into Reacti-Vials with deuterochloroform (99.98% isotopical purity) which had been molecularly distilled from a mixture of molecular sieve (3 Å) and aluminium oxide (activity I).

For quantitation of the isolated components and peak area calibration, pure samples of α-pinene, 1,8-cineol, and β-caryophyllene were used as external standards. Solutions of the individual components were concentrated by slow evaporation of the solvent in an ultra-high-purity nitrogen atmosphere and sealed off in precision 1.7-mm micro sample tubes for nuclear magnetic resonance spectroscopy (NMR). [$^1$H]- and [$^{13}$C]NMR spectra were recorded at 34°C for the isolated components on a Varian FT80 pulsed FT NMR spectrometer using the deuterochloroform mentioned above as solvent. The $CDCl_3$ also served as internal reference with $\delta_C = 77.02$. In the proton spectra, the residual $CHCl_3$ ($\delta_H = 7.24$) was used as internal reference.

A carbon disulfide extract of the leaves of the host plant *Citrus limon* (cultivar Eureka) was obtained by homogenizing 2.05 g of leaf material with 10 ml of $CS_2$ (Merck, spectroscopic grade) for 3 min using an Ultra-Turrax homogenizer. The resulting material was centrifuged and the bright green $CS_2$ extract removed from the insoluble material with a syringe. This extract was concentrated and used for GC analysis without further purification. An extract of the leaves of fennel, *Foeniculum vulgare* L., another host plant, was similarly prepared.

*Caryophyllene Oxide (I).* To a magnetically stirred solution of 1.60 g of β-caryophyllene (technical grade) in 15 ml of ethanol-free chloroform at 0°C, a solution of 1.93 g of *m*-chloroperbenzoic acid (Merck, containing 30% of *m*-chlorobenzoic acid) in 25 ml of chloroform was added from an infusion pump over a period of 30 min, after which the ice bath was removed and the reaction mixture allowed to reach room temperature. The precipitated *m*-chlorobenzoic acid was filtered off, and the filtrate was cooled to −30°C to allow further *m*-chlorobenzoic acid to crystallize. By repeating this procedure of diluting the product with a suitable solvent, cooling the resulting solution to −30°C, filtering off the precipitated *m*-chlorobenzoic acid, once more with chloroform and once with hexane as solvent, and finally removing the solvent with a rotary

TABLE 1. [$^{13}$C]NMR DATA OBTAINED FOR β-CARYOPHYLLENE ISOLATED FROM OSMETERIAL SECRETION OF *Papilio demodocus*, SYNTHETIC β-CARYOPHYLLENE, AND SYNTHETIC CARYOPHYLLENE OXIDE[a]

| Carbon[b] | Natural β-caryophyllene[c, d] ($CDCl_3$) | Synthetic β-caryophyllene[d, e] (45% v/v in $CDCl_3$) | Synthetic caryophyllene oxide (45% v/v in $CDCl_3$) |
|---|---|---|---|
| 1 | – | 53.68 d | 50.85 d |
| 2 | 28.39* | 28.42 t* | 27.22 t |
| 3 | 40.47** | 40.46 t** | 39.16 t* |
| 4 | – | 135.18 s | 59.67 s |
| 5 | 124.39 | 124.47 d | 63.55 d |
| 6 | 29.40* | 29.44 t* | 29.87 t** |
| 7 | 34.87 | 34.86 t | 30.15 t** |
| 8 | – | 154.53 s | 151.66 s |
| 9 | 48.59 | 48.57 d | 48.70 d |
| 10 | 40.04** | 40.04 t** | 39.79 t* |
| 11 | – | 32.96 s | 33.93 s |
| 12 | 30.07 | 30.06 q | 29.87 q |
| 13 | 22.67 | 22.65 q | 21.63 q |
| 14 | 111.60 | 111.71 t | 112.73 t |
| 15 | – | 16.27 q | 16.96 q |

[a]At 20 MHz, TMS as internal standard, 34°C, chemical shifts accurate to within ±0.06 ppm, * and ** denote interchangeable assignments.
[b]Numbering as in Structure I.
[c]$CDCl_3$ with $\delta_c$ = 77.02 ppm from TMS used as internal reference. Due to the small quantity of material isolated, it was impossible to observe all the lines or to determine multiplicities. A pulse angle of 48° and a pulse repetition time of 0.82 sec were used. A total of 375 000 transients were accumulated.
[d]Data tabulated for the minimum strain conformer only (Shirahama et al., 1981).
[e]Applying quantitative [$^{13}$C]NMR techniques, the population of this conformer was calculated to be 75%.

evaporator, the crude caryophyllene oxide (1.39 g, 80%) was obtained as a colorless liquid, which was further purified by a bulb-to-bulb distillation at 120°C (air bath temperature)/2 × $10^{-5}$ mm and crystallization from methanol to give caryophyllene oxide (I) as colorless crystals, mp 63–63.5°C [Ramage and Whitehead (1954) found mp 63–64°C]. This material was subjected to a detailed analysis of its [$^{13}$C]NMR spectrum (Table 1).

## RESULTS AND DISCUSSION

Larvae of the Papilionidae usually have five instars (Van Son, 1949), although it is claimed that *P. aegeus* has four (Seligman and Doy, 1972). By ab

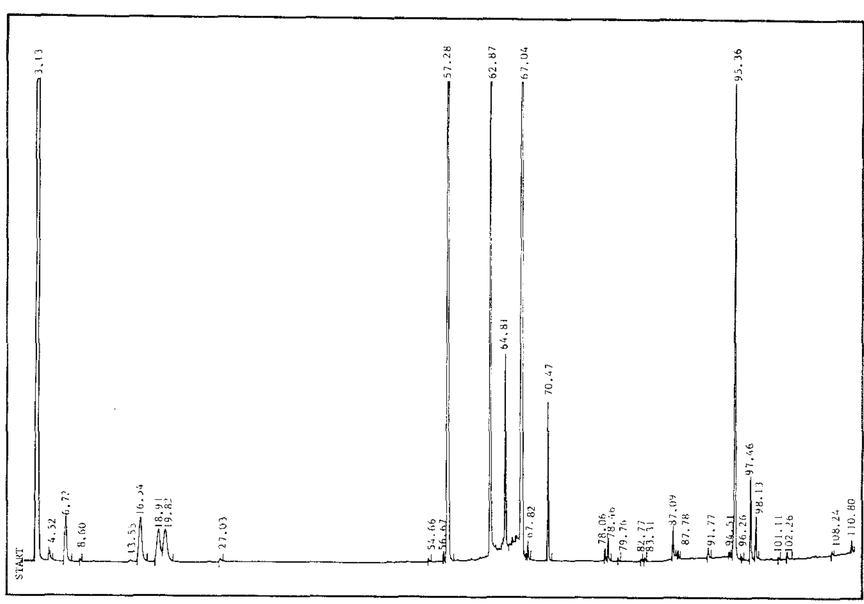



FIG. 2. Gas chromatogram of the osmeterial secretion of a fourth-instar *Papilio demodocus* larva reared ab ovum on *Citrus limon* (cultivar Eureka). Glass capillary column coated with Carbowax 20 M (40 m × 0.3 mm, film thickness 0.3 $\mu$m); 30° (20 min)–220°C at 2°C/min.

ovum rearing of the larvae of *P. demodocus*, it was determined that *P. demodocus* has five larval instars.

Regular collection and GC analysis (solventless sample introduction) of osmeterial secretion of individual larvae, showed that a rather complex mixture with a strong terpenoid-like odor was produced throughout the first four instars. A typical GC of the osmeterial secretion of a nonfinal instar larva is shown in Figure 2. With the fourth ecdysis and the concurrent changing of the black and white color pattern of the larvae to black and green, the composition of the secretion changes to that described in our previous paper (Burger et al., 1978). Immediately after the fourth ecdysis, the osmeterium was not very productive, but it was nevertheless found that the exudate contained barely detectable traces of the major components present in the secretion of the fourth instar. After about 24 hr, the secretion was produced more readily and no indication of the presence of these pre-final instar components could be found.

GC analyses of a large number of samples, collected individually from second-, third-, and fourth-instar larvae, showed that all larvae produced qualitatively similar secretions. However, these analyses also revealed considerable quantitative differences in the composition of the secretions, the most striking difference being the extremely low concentration of a group of constituents, subsequently identified as monoterpenes, in a few of the samples. Several ex-

planations, such as the effect of the sex of the larvae, its diet, its larval instar, and the possible influence of the larval color form on the composition of the secretion, were considered.

Two types of coloration patterns are found in larvae of *P. demodocus*. Larvae with the so-called umbelliferous pattern are dark green to brown with several parallel rows of small lighter colored spots over their entire length. These larvae retain a spotted appearance in the final instar, but the lighter spots acquire a light orange–red color. During the first four instars, the more common citrus color form larvae are black and have a broad white V mid-dorsally with its base extending across the posterior dorsal area. This color pattern changes to green with a black V at the fourth molt. The unbelliferous pattern is found in larvae feeding on species of Umbelliferae, while larvae feeding on *Citrus* and other species of Rutaceae have the citrus pattern (Van Son, 1949), and it has been suggested that these color forms are developed for camouflage. However, Clarke et al., (1963) have shown that the larval colors are not necessarily diet related, but determined by a single pair of genes, the gene responsible for the umbelliferous pattern being the dominant one. To determine the effect of this polymorphism and other factors on the composition of the osmeterial secretion, a large number of larvae of both color forms were reared individually in separate containers and their secretion analyzed regularly. The sex of each larva was determined by sexing the resultant adult butterflies. No correlation between the sex, instar, or color form, and the qualitative and quantitative composition of the secretion could be found, however.

Since the composition of the osmeterial secretion could conceivably also be influenced by the diet of the larvae, the presence in the secretion of a number of terpenoids that are also found in citrus leaves prompted us to compare the secretions of larvae reared on two different host plants. For this purpose a number of larvae were reared ab ovum on citrus leaves (*Citrus limon*, cultivar Eureka) and others on fennel (*Foeniculum vulgare*). Citrus foliage contains a large number of mono- and sesquiterpenes, and some monoterpenes have been identified in fennel (Trenkle, 1972), but no reference to the presence of sesquiterpenes in this plant could be found in the literature; neither could we detect these compounds by GC-MS analysis in an extract of fennel. Thus, from the obvious similarity of the GC analyses shown in Figures 2 and 3, it does not seem likely that the quantitative composition of the secreted material is influenced by the diet of the larvae, or that the sesquiterpenoid constituents, at least, are obtained directly from the host plant. This observation is in agreement with the conclusion reached by Honda (1983b) that the terpenoids in the osmeterial secretion of *P. protenor demetrius* and *P. helenus nicconicolens* are not obtained from the host plant, *Fagara ailantoides*. In an earlier study it had also been found that $\beta$-selinene and selin-11-en-4$\alpha$-ol, which are present in the osmeterial secretion of *Battus polydamas*, are not detectable in its host plant (Eisner et al., 1971).

As the experiments discussed so far did not explain satisfactorily the ab-

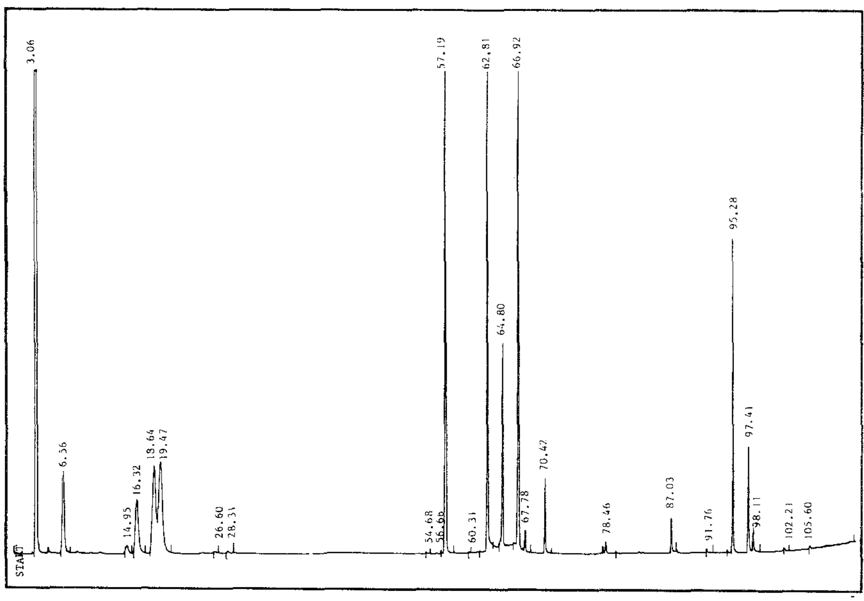
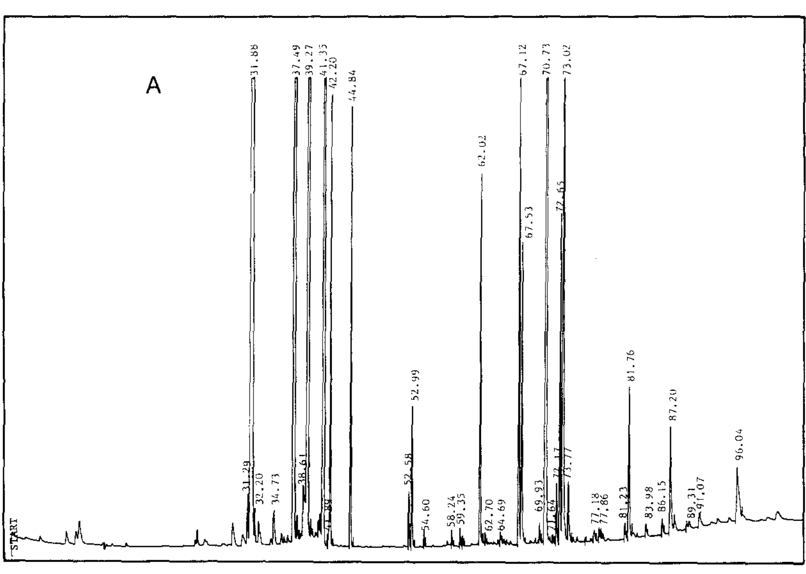



FIG. 3. Gas chromatogram of the osmeterial secretion of a fourth-instar *Papilio demodocus* larva reared ab ovum on *Foeniculum vulgare*. GC parameters as in Figure 2.

sence of the monoterpenoids from some of the samples that were analyzed, other variables, such as the number of times material was collected from individual larvae, had to be considered. To increase the possibility of obtaining a satisfactory analysis for a specific larva at a specific stage, material was collected separately from the two prongs of the osmeterium for duplicate analyses. From the results of a set of these duplicate analyses, it became clear that, in a few instances, quantitative differences also existed between samples collected from the two prongs of the osmeterium of the same larva, as is illustrated by two gas chromatograms shown in Figure 4A and B. Although this phenomenon is still under investigation, it therefore seems possible that the composition of the secretion may be influenced by the extent to which a larva is irritated. Normally larvae react to the slightest irritation by readily everting the osmeterium to about half its full length, whereafter, on prolonged or more intense irritation, it is extended to its full length. The tips of the two prongs of the osmeterium are generally noticeably darker than the rest of the organ and, as working hypothesis, it is now assumed that quantitatively different material could be secreted at different stages of osmeterium eversion or by different parts of the osmeterium.

*Chemical Analysis.* GC-MS analyses, carried out with columns having different polarities, different temperature programs, etc., furnished mass spectra for all of the components in the secretion. Figure 5 shows a typical mass chro-



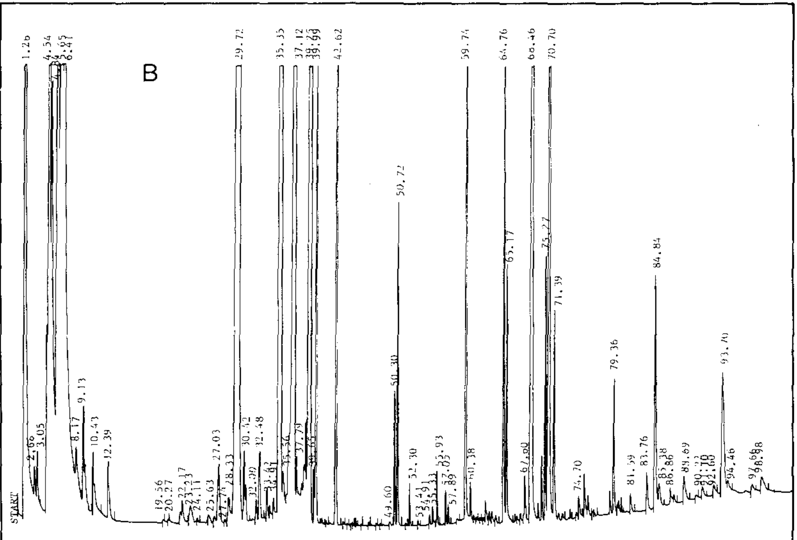




FIG. 4. Gas chromatographic comparison of the secretions (A and B) collected separately from the two prongs of the osmeterium of a *Papilio demodocus* larva. Glass capillary column coated with Carbowax 20 M (40 m × 0.3 mm, film thickness 0.3 μm); 40–220°C at 2°C/min.

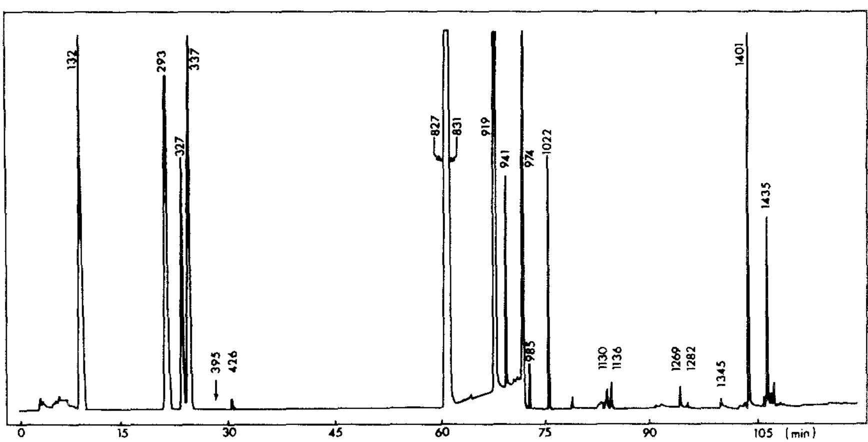



FIG. 5. Mass chromatogram (dedicated mass chromatogram plot: *m/z* 90–230) for the osmeterial secretion of pre-final instar *Papilio demodocus* larvae. GC parameters as in Figure 2.

matogram obtained from the data of such an analysis by plotting the sum total of the ion intensities for the mass range *m/z* 90–230 versus spectrum number. This mass range was chosen in order to eliminate the solvent, dichloromethane, from the chromatogram.

Components 132, 293, 327, 337, 395, and 426 were identified as α-pinene, myrcene, limonene, β-phellandrene, (*Z*)-ocimene, and (*E*)-ocimene, respectively, by comparison of their mass spectra with published data (Stenhagen et al., 1974) and by retention time comparison with synthetic samples. All of these monoterpenes have been found in the osmeterial secretion of *P. machaon hippocrates*, *P. maackii tutanus*, and *P. protenor demetrius*, while in addition to these compounds the secretion of *P. bianor dehaanii* also contained sabinene.

In Figure 5, the two components 827 and 831, having distinctly different mass spectra, appeared as one broad peak. Although many sesquiterpenes have almost identical mass spectra, components 827 and 831 could tentatively be identified as β-elemene and β-caryophyllene respectively, by comparison of their mass spectra with published data (Stenhagen et al., 1974). Preparative GC on a packed Carbowax 20 M column, on which these two components were fully separated, yielded practically pure samples of components 827 (73 μg) and 831 (280 μg) for NMR analysis. Except for the presence of a resonance at δ ca. 1.55 which was found to be present in all the [$^{1}$H]NMR spectra of micro samples, and which arises from moisture introduced into the samples during sample preparation, the [$^{1}$H]NMR spectra of components 827 and 831 were practically identical to that of synthetic samples of β-elemene and β-caryophyllene, respectively.

The identification of component 831 as β-caryophyllene was further substantiated by the excellent agreement of its [$^{13}$C]NMR spectrum with that of a

synthetic sample. The [$^{13}$C]NMR data obtained for the isolated material are given in Table 1 together with the $^{13}$C resonances of a synthetic sample of *β*-caryophyllene. As the multiplicities given and the assignments made by Bohlmann and Zdero (1978), Shirahama et al., (1981) and Formáček and Kubeczka (1982) were found to be inconsistent, the [$^{13}$C]NMR spectrum of *β*-caryophyllene together with that of caryophyllene oxide was reinvestigated. Multiplicities were determined by applying the APT technique (Patt and Shoolery, 1982). Integration of the [$^{13}$C]NMR spectrum of *β*-caryophyllene (at 34°C), under conditions of gated decoupling (without NOE) and in the presence of $Cr(AcAc)_3$ (0.1 M), revealed that the population of the two major minimum strain conformers was 75% and 25%, respectively. The assignments given in Table 1 were verified by calculating the HOSE code for each carbon atom and referring to [$^{13}$C]NMR shift range tables (Bremser et al., 1982).

Component 919 was identified as (*E*)-*β*-farnesene by comparison of its [$^{1}$H]- and [$^{13}$C]NMR, and mass spectra, as well as its retention time, with data obtained for synthetic (*E*)-*β*-farnesene. The preparatively isolated sample contained approximately 10% of a second component which was positively identified as *β*-elemene by its retention time and [$^{1}$H]NMR spectrum, obtained by subtraction of the spectrum of (*E*)-*β*-farnesene from that of the isolated sample. The presence of *β*-elemene in the isolated *β*-farnesene sample will be discussed below, together with evidence from which it became clear that component 974 was germacrene-A.

Components 941, 985, 1022, 1130, 1136, 1269, 1282, and 1345 all gave typically terpenoid mass spectra, but they remained unidentified as preparative GC afforded insufficient material for NMR analysis.

From retention time data, component 1401 was concluded to be a sesquiterpene alcohol. Its mass spectrum (Figure 6) exhibited a weak molecular ion at *m/z* 220 (0.5%) and fragment ions at *m/z* 202 (4%) and *m/z* 205 (3%) which can be attributed to the loss of water and a methyl radical, respectively, from

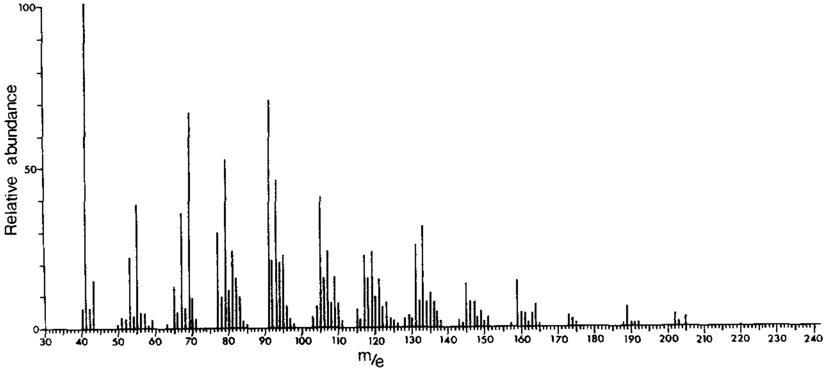
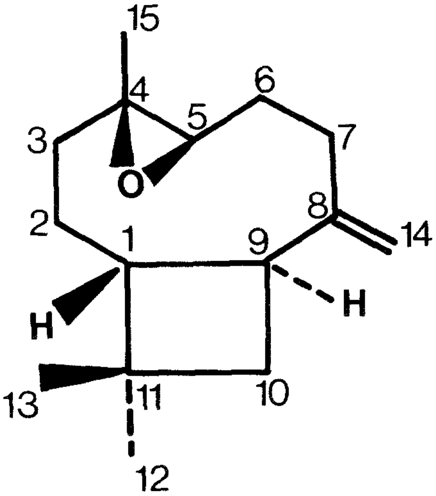



FIG. 6. Mass spectrum of component 1401.

the molecular ion. The [$^1$H]NMR spectrum confirmed the presence of a hydroxymethyl group, as well as some of the structural features of $\beta$-caryophyllene, such as the presence of two methyl groups on a quarternary carbon atom ($\delta$ 0.94 and 0.96), and olefinic methylene group ($\delta$ 4.77 and 4.61), and a proton on a trisubstituted double bond ($\delta$ 5.61). The magnitude of the vicinal coupling $^3J_{HH}$ of olefinic protons with allylic methylene protons in cyclic compounds, having one double bond in the ring, is ring size dependent; a coupling of 8.2 Hz being expected for (*Z*)-cyclononene (Sternhell, 1969). The observed coupling constant $^3J_{HH}$ = 8.1 Hz between the olefinic proton at $\delta$ 5.61 and the methylene protons therefore, may, be taken as evidence that, if this double bond is present in a ring, the ring contains more than seven carbon atoms. The compound, therefore, apparently contains an allyl alcohol function; the hydroxymethyl allylic protons of this function resonate at $\delta$ 4.06. The [$^{13}$C]NMR spectrum of this component, although very weak, due to the low sample concentration, exhibited a line at $\delta$ 30.07 which is typical for the C-12 methyl of a $\beta$-caryophyllene type of structure (numbering as in Structure I). The absence of further information on its NMR spectra, however, precluded the proposal of an acceptable structure for this component.




STRUCTURE I.

Component 1435 was assumed to be an isomer, possibly the *E* isomer, of component 1401, as their mass spectra were practically identical, except for small differences in the relative abundance of some ions. As the preparative gas chromatographic separation yielded a very small quantity of impure material, NMR spectroscopy did not furnish further structural information, and this component also remained unidentified.

The possibility that one of these two compounds could be caryophyllene oxide, found in *Papilio* species by Honda (1980a, 1981), was also investigated.

Synthetic caryophyllene oxide (I) was prepared for this purpose by epoxidation of β-caryophyllene with *m*-chloroperbenzoic acid. The epoxide thus prepared (Structure I) was characterized by comparison of its [$^{13}$C]NMR spectrum (Table 1) with published spectra (Bohlmann and Zdero, 1978; Shirahama et al., 1981; Formáček and Kubeczka, 1982). From a comparison of the mass spectrum of the synthetic epoxide, obtained by GC-MS (Figure 7) with that of component 1401 (Figure 6), it is clear that this component cannot be caryophyllene oxide (I). As expected, the oxide also had a shorter retention time than that of the alcohol 1401. The doublet of doublets (δ 2.88) of the proton on C-5 of caryophyllene oxide was absent in the [$^{1}$H] spectrum of component 1401.

Gas chromatographic analyses of the osmeterial secretions on Carbowax 20 M usually show a broad pre-tailing peak at a retention time between that of component 831 (β-caryophyllene) and component 974. This material, eluting as a broad peak, had a mass spectrum similar to that of β-elemene. The presence of the β-elemene in the β-farnesene sample, which was mentioned earlier, cannot be attributed to tailing of the β-elemene, since the β-farnesene should then also contain β-caryophyllene, which is present in the secretion in a high concentration and is eluted closer to β-farnesene. The determination of the stability of the osmeterial secretion under different GC conditions produced information on which a plausible explanation for the presence of the broad peak in the sesquiterpene region, and the presence of β-elemene in the isolated β-farnesene, could be based.

It is known that even at low temperatures, β-elemene is readily formed from germacrene-A by a Cope rearrangement (Weinheimer et al., 1970). Therefore, experiments were carried out to determine whether β-elemene is secreted by the larvae or whether it is formed from a precursor in the hot inlet of the GC. A thick film SE-30 column, on which β-elemene was fully separated from the other sesquiterpenes, was used for these experiments. With the injector pro-

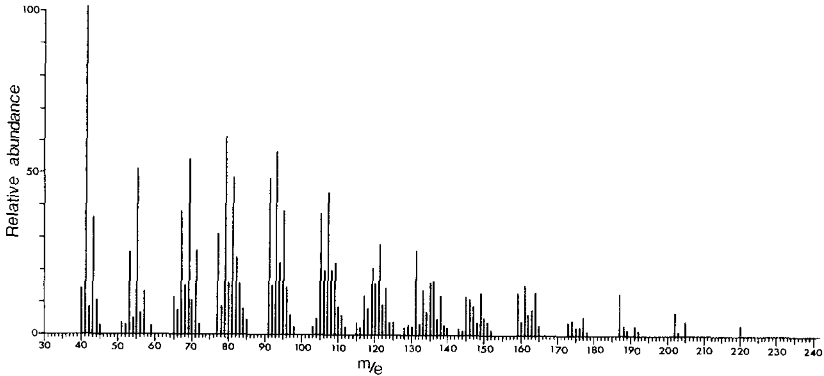


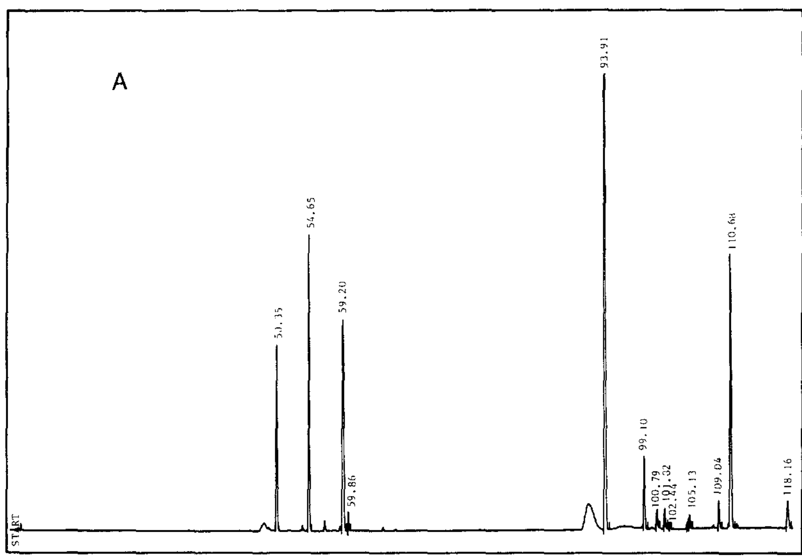

FIG. 7. Mass spectrum of caryophyllene oxide obtained by GC-MS analysis of a synthetic sample.

gramed from 50 to 200°C at 40°C/min, the β-elemene peak did not appear in the resulting GC trace shown in Figure 8A, whereas at an injector temperature of 190°C, β-elemene is formed in the injector and is eluted as a sharp peak (Figure 8B). In spite of the appearance of other small peaks at the higher injector temperature, which complicated such a correlation, it was clear that the formation of β-elemene could not be accounted for by a comparable decrease in the peak area of one of the other sharp peaks in the sesquiterpenoid region of the gas chromatogram. However, it was found that at an injector temperature of 240°C, the broad peak between the β-elemene and β-caryophyllene peaks had disappeared, and the reasonable assumption was therefore made that this broad peak could be attributed to a thermally labile precursor of β-elemene being present in the secretion. The GC trace in Figure 8B can therefore be explained by assuming that, after incomplete isomerization in the injector, a mixture of the precursor and its product is transported onto the cold column, after which the precursor isomerizes on the column at temperatures approaching the elution temperature of β-elemene (ca 210°C), resulting in sharp and broad peaks for the β-elemene formed in the injector and on the column, respectively.

As expected, the mass spectra obtained for each of these two peaks were identical in every detail. From similar observations made by Weinheimer et al. (1970) on the isomerization of germacrene-A to β-elemene in the GC injector, it seemed reasonable to conclude that germacrene-A is the precursor from which β-elemene is formed. The sesquiterpenes were eluted at temperatures approximately 90°C lower on the Carbowax 20 M column than on the SE-30 column, and at an elution temperature of ca. 120°C germacrene-A, as well as some rearrangement to β-elemene, could be observed on the Carbowax 20 M column. These conclusions were confirmed by the absence of any detectable rearrangement when the secretion was subjected to GC on a 15-m Carbowax 1000 column, from which germacrene-A was eluted below 100°C.

In the gas chromatogram shown in Figure 9, a broad peak is observed in the monoterpene region. By GC-MS analysis and retention time comparison with an authentic synthetic sample, this component was identified as 3-hydroxybutanoic acid. With Carbowax 20 M as stationary phase, this component could barely be observed as it is eluted as a very broad band. By plotting a mass chromatogram for the ion $m/z$ 60, and by comparison of retention time data, it was found that this hydroxy acid elutes at a retention time comparable to that of component 1269 in Figure 5. Due to its high solubility in water, the hydroxy acid was present only in small concentrations in the dichloromethane extracts which were used for GC and GC-MS analyses. The identification of 3-hydroxybutanoic acid prompted us to look for esters of this acid in the secretion. GC and GC-MS analyses were carried out on an OV-73 capillary column and methyl 3-hydroxybutanoate was found to be present in low concentrations in some samples. A typical GC with OV-73 as stationary phase is given in Figure 9.

Finally, to obtain results from which a picture of the average quantitative



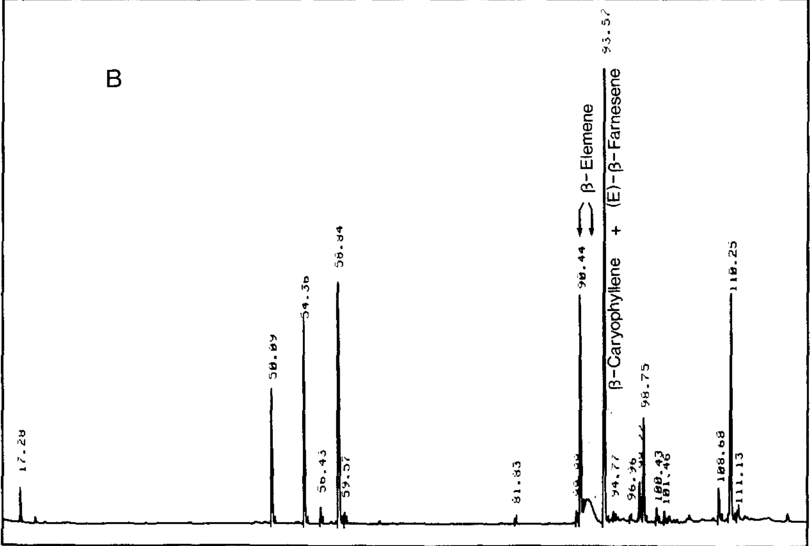




FIG. 8. GC analysis of the osmeterial secretion of pre-final instar *Papilio demodocus* larvae on a glass capillary column coated with SE-30 (40 m × 0.3 mm, film thickness 3.0 μm); 30–250°C at 2°C/min. (A) Injector programed 50–200°C at 40°C/min; (B) injector isothermal at 190°C.

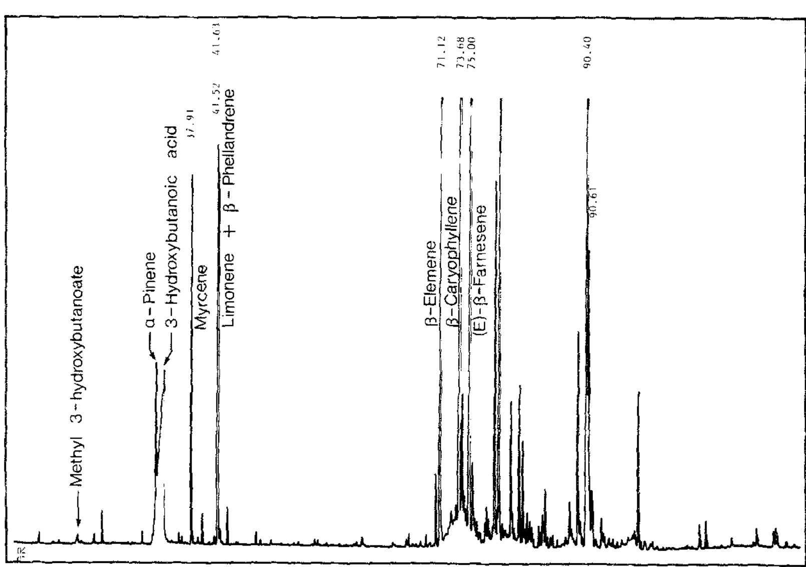



FIG. 9. GC analysis of the osmeterial secretion of pre-final instar *Papilio demodocus* larvae on a glass capillary column coated with OV-73 (40 m × 0.3 mm, film thickness 1.0 μm); 30–250°C at 2°C/min; injector isothermal at 230°C.

composition of the secretion could be formed, the osmeterial secretions of more than 200 second-, third-, and fourth-instar larvae were combined, extracted with deuterobenzene, and analyzed on the Carbowax 20 M, Carbowax 1000, and OV-73 capillary columns. Quantitative information obtained from all three chromatograms were used for the calculation of the quantitative composition of this sample. The relevant data are given in Table 2. Due to the expected incomplete extraction of 3-hydroxybutanoic acid and its methyl ester from the water layer, peak areas for these two constituents were not taken into consideration in the calculations. However, an indication of their respective concentrations in the secretion can be obtained from further data included in the table to illustrate the enormous variation in the concentration of the secretions from different larvae. As no internal standard was used, the integrated peak areas were normalized with respect to β-farnesene which, although its concentration undoubtedly also varied, nevertheless proved to be stable under the GC conditions employed and was fully separated from other constituents on these columns. The figures for germacrene-A were obtained from the broad β-elemene peak in GC analyses using SE-30 as stationary phase.

The ratio of organic material to water in the secretion was not determined accurately, but from measurements of the volume of the oil that separated out on top of the water layer in the collecting capillaries, it was concluded that the secretion contained approximately 5% of organic material.

TABLE 2. COMPOSITION OF VOLATILE ORGANIC FRACTION OF OSMETERIAL SECRETION OF PRE-FINAL INSTAR *Papilio demodocus* LARVAE

| Peak no. in Figure 5 | Composition (%) | Concentration range[a] | Component | Mass spectral data $m/z$ (abundance, %) |
|---|---|---|---|---|
| | | 0–2 | Methyl 3-hydroxybutanoate[b] | $M + 1^+$: 119(2), 103(25), 100(7), 87(20), 74(57), 71(32), 69(13), 61(14), 59(14), 49(18), 45(49), 43(100) |
| | | 4–500 | 3-Hydroxybutanoic acid[b] | $M^+$: 104(0), 89(13), 86(5), 71(38), 61(2), 60(42), 58(5), 45(80), 44(52), 43(100), 42(43), 41(11) |
| 132 | 1.1 | 0–470 | α-Pinene | $M^+$: 136(8), 121(14), 107(6), 105(11), 94(9), 93(100), 92(32), 91(31), 80(8), 79(18), 77(25), 67(8), 65(4), 55(5), 53(8), 51(2), 43(3), 41(10), 39(7) |
| 293 | 3.0 | 0–705 | Myrcene | $M^+$: 136(2), 121(2), 93(57), 91(11), 80(7), 79(10), 77(9), 69(59), 67(8), 53(9), 41(100), 39(17) |
| 327 | 2.0 | 0–1070 | Limonene | $M^+$: 136(16), 121(15), 107(12), 93(40), 79(16), 77(9), 68(100), 67(29), 53(10), 41(9), 39(7) |
| 337 | 1.9 | 0–1125 | β-Phellandrene | $M^+$: 136(15), 121(7), 93(100), 92(10), 91(35), 79(25), 77(40), 65(9), 53(9), 51(6), 43(10), 41(26), 39(17) |
| 395 | 0.1 | 0–5 | (*Z*)-Ocimene | $M^+$: 136(2), 121(12), 105(15), 93(100), 92(35), 91(45), 80(25), 79(28), 77(28), 67(11), 65(5), 55(5), 53(9) |
| 426 | 0.3 | 0–30 | (*E*)-Ocimene | $M^+$: 136(9), 121(16), 107(8), 105(22), 93(100), 92(23), 91(35), 80(48), 79(40), 77(38), 67(16), 55(10), 43(7), 41(32), 39(12) |

| | | | | |
|---|---|---|---|---|
| 831 | 25.3 | 680–2480 | β-Caryophyllene | $M^+$: 204(9), 189(15), 175(7), 161(29), 148(25), 147(26), 133(73), 121(28), 120(38), 119(33), 109(21), 107(41), 105(48), 93(100), 91(68), 81(42), 79(68), 77(32), 69(97), 67(38), 55(42), 53(25), 41(95) |
| 919 | 18.1 | 1000 | (*E*)-β-Farnesene | $M^+$: 204(10), 161(21), 133(43), 120(31), 119(12), 109(13), 107(16), 105(14), 93(97), 91(25), 81(38), 79(39), 77(19), 69(100), 67(38), 55(37), 53(22), 41(98) |
| 941 | 3.4 | 330–1080 | Unidentified | $M^+$: 204 |
| 974 | 21.3 | 240–1350 | Germacrene-A[c] | $M^+$: 204(3), 189(28), 161(33), 147(38), 133(28), 121(47), 119(28), 107(58), 93(92), 81(100), 79(45), 68(64), 67(47), 55(30), 53(25), 41(37) |
| 985 | 0.8 | 40–120 | Unidentified | $M^+$: 204 |
| 1022 | 3.2 | 110–390 | Unidentified | $M^+$: 204 |
| 1130 | 0.3 | 10–40 | Unidentified | $M^+$: 220 |
| 1136 | 0.6 | 25–105 | Unidentified | $M^+$: 220 |
| 1269 | 1.2 | 10–90 | Unidentified | $M^+$: 218 |
| 1282 | 0.6 | 5–300 | Unidentified | $M^+$: 218 |
| 1345 | 1.1 | 10–470 | Unidentified | $M^+$: 220 |
| 1401 | 9.7 | 500–1940 | Unidentified | $M^+$: 220 |
| 1435 | 2.5 | 35–460 | Unidentified | $M^+$: 220 |
| | 3.6 | | Other minor components | |

[a]Concentrations calculated by using results from the GC analyses of the osmeterial secretions of individual larvae. An arbitrary concentration of 1000 was assigned to (*E*)-β-farnesene and the other concentrations were normalized with respect to this component.
[b]Observed in GC analyses with OV-73 as stationary phase.
[c]Partially or fully converted to β-elemene in the hot GC-MS interface.

A program is currently being undertaken to collect a large number of larvae during the next few seasons in order to obtain more structural information on the still-unidentified, minor components. The ecological function of the osmeterial secretion of the pre-final instar larvae was not investigated in the present study, but from observations in citrus orchards and in the laboratory, it appears that active *P. demodocus* larvae with intact osmeteria (even if these are not very productive) are not attacked by the indigenous ant species, for example *Crematogaster peringueyi* and *Pheidole capensis* or by the introduced *Iridomyrmex humilis*, in agreement with the results obtained by Honda (1983a) in his work on the defensive potential of components of the larval osmeterial secretion of papilionid butterflies against ants.

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# MANDIBULAR GLAND SECRETIONS OF TWO PARASITOID WASPS (HYMENOPTERA: ICHNEUMONIDAE)

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**Abstract**—Males of *Rhyssa persuasoria* and *Megarhyssa nortoni nortoni* exhibit marked aggregation behavior prior to and during the emergence of females from host trees, and this has been linked with the secretion of an odorous liquid from the mandibular glands. The volatile components of these secretions were examined by combined gas chromatography–mass spectrometry. While both species contained 6-methylhept-5-en-2-one, *M. nortoni nortoni* was characterized by a series of alkyl spiroacetals and *R. persuasoria* contained 3-hydroxy-3-methylbutan-2-one. The same spiroacetals have previously been isolated from the mandibular glands of other Hymenoptera and have been directly associated with aggregation behavior in some species. The chemical and behavioral aspects of the two species are discussed.

**Key Words**—Mandibular secretions, parasitoids, aggregation pheromones, spiroacetals, 6-methylhept-5-en-2-one, 3-hydroxy-3-methylbutan-2-one, *Rhyssa, Megarhyssa,* Hymenoptera, Ichneumonidae.

## INTRODUCTION

The parasitoids *Megarhyssa nortoni nortoni* Cresson (hereafter referred to as *M. nortoni*) and *Rhyssa persuasoria* L. (Hymenoptera: Ichneumonidae) were introduced into Australia as natural enemies of the wood wasp, *Sirex noctilio* F., an exotic pest of radiata pine (*Pinus radiata* D Don) (Taylor, 1967). Both species attack mature larvae of *S. noctilio* within the wood of infested trees to emerge, in general, 12 months later.

Males of both species aggregate at sites of potential emergence or at sites



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which are otherwise attractive. The aggregation response is initiated by volatiles produced by the symbiotic fungus of *S. noctilio, Amylosterum areolatum* (Madden, 1968). Localized generation of fungal volatiles is presumably stimulated by moisture changes associated with adult eclosion, since injection of water into fine drill holes made in dry, fungus-infested logs will promote aggregation at such sites after 24–48 hr (Madden, unpublished results).

The aggregation behavior of male *M. nortoni* is similar to that described for several other *Megarhyssa* species (Heatwole et al., 1963; Crankshaw and Matthews, 1981), and the group is typically compact with arching of the abdomen so that the distal abdominal segments make contact with the bark substrate at the point of interest discerned by the antennae. Aggregations of *R. persuasoria* are less compact and males are incapable of arching their abdomens, although curvature of the abdomen may be observed.

In laboratory studies with *M. nortoni* males, presentation of the fungus on agar or water extracts of insect frass is sufficient to initiate individual interest followed by, through 4-min exposure periods, accelerated recruitment of males to the sites. This second response is a function of the number of males making contact with each other (Madden, unpublished results). In this situation mutual antagonism occurs between individuals, in which opening of the mandibles is observed. Furthermore, the rapid drumming of the antennae at the sites of emerging insects elicits a like response in the emergent. In the majority of cases, the space between the mandibles is observed to be occupied by a liquid.

Depending on the species of the emergent, a number of outcomes may occur: (1) If the emergent is a nonparasitoid, e.g., *S. noctilio,* the male aggregation of either parasitoid is retained and males will mount the emergent and display precopulatory movements. (2) If the emergent parasitoid species is a conspecific, of either sex, precopulatory activity is enhanced and mounting occurs. (3) If the emergent parasitoid species is dissimilar, the males' precopulatory activity, which may include males mounting males, will subside and the aggregation will disperse.

The odor of each species is characteristically different and, in preliminary experiments, it was found that it could be released if the insects were grasped or shaken. In addition, mandibular opening with the appearance of liquid could be elicited by swift decapitation. The odor source was clearly localized in the head, as crop and stomach contents and thorax and abdomen possessed no such odor.

It would appear that this secretion plays an important role in the complex species recognition, aggregation formation, and defensive behavior of the two species. This paper reports the chemical identity of the major volatile compounds occurring in the mandibular secretion of male and female *R. persuasoria* and *M. nortoni*. Compound classes encountered include spiroacetals, ketones, alcohols, and esters.

## METHODS AND MATERIALS

*Insect Material.* Insects were collected as they emerged from infested logs in an insectary, were kept in polystyrene pots, provided with water and honey, and held at 10°C between tests. They were then available for bioassy or chemical examinations as required.

Three main approaches were used in the study of the volatiles. These were the direct collection of the secretions from stimulated individuals, examination of extracts of dissected mandibular glands to confirm the origin of the secretion, and the examination of extracts of excised heads. Males and females were treated separately.

*Collection of Secretions.* The mandibular secretions were collected in the following manner. Individual insects were grasped at the thorax between thumb and forefinger, an action which resulted in the opening of the mandibles and the appearance of the secretion in variable amounts. The tip of a 1-$\mu$l microcapillary tube (Microcaps, Drummond Scientific Co., Broomall, Pa.) was placed between the extended mandibles resulting in the uptake of the liquid. The microcap was either immediately dropped into, or its contents flushed into, a vial of pentane. Maximum yield per collection ranged from 0.25 $\mu$l to 0.80 $\mu$l for *R. persuasoria* and *M. nortoni*, respectively. In general greater quantities were obtained from females and *M. nortoni*. Insects were returned to a new container, stored at 10°C, and could be processed daily for up to 14 days.

*Gland Material.* Mandibles and associated glands, gland reservoirs, and muscles were dissected from parasitoids that had been killed by short exposure to −20°C prior to analysis. Ten to fourteen mandibles for each species and sex were prepared. In another approach, insects were decapitated, antennae removed, and the heads stored in pentane.

*Extraction.* The frozen gland material and heads were crushed and left in pentane overnight. The gland extract was filtered and used without further work-up after evaporation to minimum volume. The head extracts were washed with 5% sodium bicarbonate solution to remove fatty acids, dried over $MgSO_4$, and evaporated to a minimum volume prior to injection.

*Bioassay.* The response of male parasitoids to live females contained in perforated vials, excised female heads, and mandibular secretion was evaluated. In paired comparisons, presentation of a test subject to 25 males of one species was alternated with a similar presentation to 25 males of the second species through 10 presentations for each of the three species treatments. The number of males that visited or revisited and drummed the test site with their antennae during a 2-min exposure was recorded to evaluate the response to each treatment.

*Gas Chromatography-Mass Spectrometry.* A Pye 204 gas chromatograph was employed, directly coupled to a VG 70/70F mass spectrometer with 2035

data system. The mass spectrometer was normally operated in the electron impact mode at 70 eV, 4 KV accelerating volts, and a source temperature of 200°C. Scans from *m/z* 350–20 were stored every 1.6 sec. Chemical ionization analyses were carried out with isobutane as the reagent gas at a pressure of approximately 1 torr in the ion chamber. Accurate masses were determined at a resolution of 1000 by means of perfluorokerosene as an internal reference with peak times relative to the reference peak times being used. The gas chromatography was carried out on 50-m × 0.2-mm fused silica OV-101 or 25-m × 0.2-mm BP1 (bonded-phase equivalent of OV-101, SGE Pty. Ltd) with the column passing through into the ion source. The carrier gas was hydrogen, with a flow rate at 70°C of 1.8 ml atm/min (Davies, 1984). Injections were splitless at 250°C, and a typical column temperature program was 70–250° at 4°/min.

The mandibular secretions inside microcapillaries were sealed inside larger capillaries (2 cm × 1 mm) and injected with a capillary crusher, a modification of a previously described device (Stanley and Kennett, 1973).

Hydrogenations were carried out in sealed Reacti-vials (Pierce) using Adams catalyst. Methylations were carried out with ethereal diazomethane. Trimethylsilylation was carried out with BSTFA (Pierce), and methoxime derivatives were prepared with methoxylamine hydrochloride (2% in pyridine).

Identifications were based on GC retention indices, mass spectra, chemical modification of functional groups and, where possible, by comparison with authentic standards.

## RESULTS

The mandibular gland secretion is presumably produced by the glandular mass of cells that envelop the mandibular gland reservoir (Figure 1). The volumetric capacity per reservoir was approximately 2.0 and 1.4 μl for average-size female and male *M. nortoni*, respectively, and 1.5 and 1.0 μl for average-size female and male *R. persuasoria.* The ducts from the reservoir pass through the mandible to open on the inner margin at the base of the mandible.

The secretions showed the presence of several volatile components, with that of *M. nortoni* being considerably more complex than that of *R. persuasoria.* Figure 2 shows the chromatogram obtained from the secretion of a single *M. nortoni* female. Peak 1 was found to be pentan-2-one, and peak 2 to be 6-methylhept-5-en-2-one. This latter compound was also readily identified in the secretion of *R. persuasoria. M. nortoni,* however, also produced a series of compounds (peaks 3–6, Figure 2), the mass spectra of which did not match any compound in the available mass spectral data bases.

Three of these compounds (peaks 3, 4, and 6) were found to have a molecular weight of 184 and prominent fragment ions at *m/z* 112 and 115, while the molecular weight of the other (peak 5) was 168. Peak 5 was not consistently

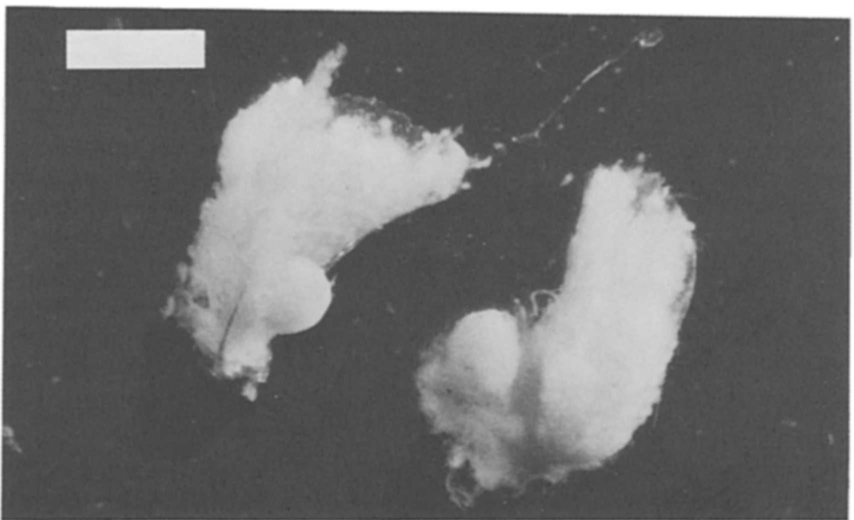

FIG. 1. Excised mandibles and associated glands and tissue from a *M. nortoni* male. Photographed in 70% ethanol. Scale represents 1 mm.

found and appeared to diminish rapidly on storage, indicating an unstable structure. The molecular weight 184 compounds were found from accurate mass determinations to be $C_{11}H_{20}O_2$ (found, 184.149; calculated for $C_{11}H_{20}O_2$, 184.146). Using bulked material from whole heads, compounds 3, 4, and 6 failed to hydrogenate or form any methyl ester, methoxime, or trimethylsilyl deriva-

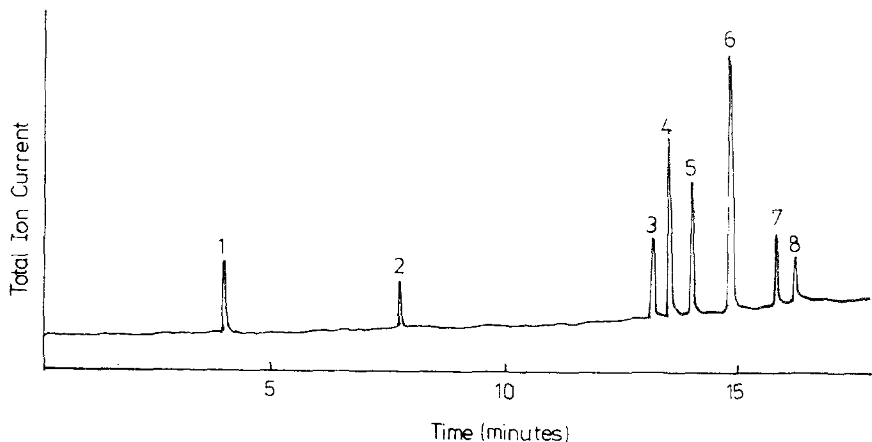


FIG. 2. Chromatogram of the volatile components in the mandibular gland secretion of a single *M. nortoni* female. GC column was 50 m OV-101, fused silica, programed from 70° to 200°C at 4°/min. Peak numbers are referred to in the text and Table 1.

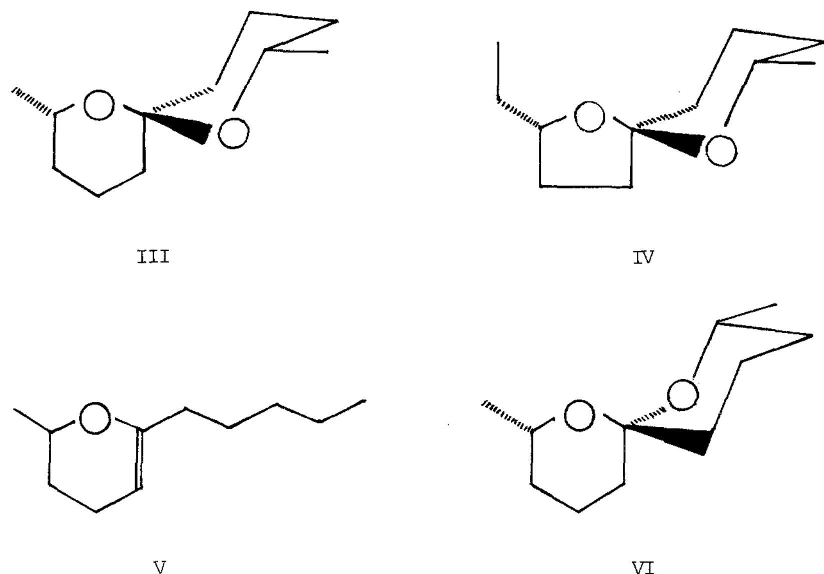


FIG. 3. Structures of characteristic *M. nortoni* compounds, with numerals corresponding to peak numbers in Figure 2. III: *E*, *E*-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane; IV: *E*,*E*-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane; V: 2-methyl-6-pentyl-3,4-dihydro-2H-pyran; VI: *Z*,*E*-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane.

tive, indicating that there were no double bonds present and that the oxygens were not present as acid, ketone, or alcohol groups. Saponification with ethanolic potassium hydroxide also effected no change, indicating that no ester function was present.

The two degrees of unsaturation, therefore, had to be accounted for by ring systems, with the oxygens in the ring, suggesting a cyclic acetal could account for the structure. An examination of the literature for known acetals of the same formula showed that the mass spectra of compounds 3, 4 and 6 did in fact match closely a series of previously reported spiroacetals (Francke et al., 1978, 1979, 1980a) from the mandibular glands of bees and wasps. Close comparison of mass spectra and gas chromatographic retention indices with those of authentic samples run under identical conditions confirmed the identities, and enabled stereoisomers to be assigned. Peak 3 was shown to be *E*,*E*-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, peak 4 to be *E*,*E*-2-ethyl-7-methyl-1,6-dioxaspiro-[4.5]decane, and peak 6 to be *Z*,*E*-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. (Figure 3)

The two stereoisomers of the synthetic dimethyl-1,7-dioxaspiro[5.5]-undecanes are well separated on nonpolar stationary phases and can be distinguished as well by their mass spectra which show significant differences (Francke

et al., 1980a). The two synthetic dimethyl-1,6-dioxaspiro[4.5] decanes (*E,E* and *Z,E* isomers) were much closer in relative retention time and had virtually identical mass spectra. Only the *E,E* and *Z,E* steroisomers of the 1,7-dioxaspiro[5.5]undecanes would appear to be thermodynamically stable (Francke et al., 1980b; Mori and Tanida, 1981). The chirality of the spiroacetals was not determined.

Peak 5 was shown to correspond to $C_{11}H_{20}O$ (found, 168.147; calculated for $C_{11}H_{20}O$, 168.151), and it appeared that it could be related to an unknown previously reported in association with the same spiroacetals (Bergström et al., 1982) which was subsequently shown to be the biogenetically related 2-methyl-6-pentyl-3,4-dihydro-2H-pyran (Francke, 1984; Francke et al., 1985). Peak 5 had a mass spectrum with *m/z* 168 (23%), 125 (42), 112 (100), 97 (30), 84 (16), 83 (31), 70 (25), 58 (15), 55 (61), and 41 (36). Comparison of this spectrum with that of the above dihydropyran (Francke, personal communication) confirmed that the spectra were very similar, and an authentic sample run under the same conditions confirmed the assignment based on an identical retention index and mass spectrum.

Peaks 7 and 8 in Figure 2 were found to be undecan-2-one and undecan-2-ol, respectively. Methyl oleate was observed as a major component in the *M. nortoni* secretion. A major peak in the *R. persuasoria* secretion gave a mass spectrum with *m/z* 31 (42%), 39 (17), 41 (28), 43 (49), 59 (100) and 87 (5). After chemical ionization mass spectrometry to obtain a molecular weight, and subsequent comparison of GC and MS data with an authentic sample, this was found to be 3-hydroxy-3-methylbutan-2-one.

The components identified in the two secretions are summarized in Table 1. Analysis of excised mandibular gland extracts confirmed that the volatiles in the secretion did in fact originate from this gland. In addition to the compounds listed, several other peaks were observed at longer retention times in *M. nortoni* which have yet to be identified. The gland and head extracts of *M. nortoni* were also found to contain normal fatty acids such as palmitoleic, palmitic, oleic, linoleic, and stearic acids and a range of cuticle wax hydrocarbons including *n*-heneicosane, *n*-tricosane, *n*-pentacosane, *n*-heptacosane, as well as a C-25 alkene, while *R. persuasoria* contained the same range of fatty acids with a number of branched-chain alkanes in addition to the *n*-alkanes.

Whole females, excised female heads, and female secretions were used in bioassays to determine the degree of attraction to conspecific males and the degree of interspecific attraction. Table 2 summarizes these results. In each comparison, a significant preference for males to respond to females of the same species, their excised heads, and in particular their mandibular secretions was apparent. Excised heads were the least effective in eliciting activity by males, followed by live females, and then mandibular secretion which was the most active and specific.

TABLE 1. PRINCIPAL COMPONENTS FOUND IN MANDIBULAR SECRETIONS[a]

| | | *M. nortoni* | | *R. persuasoria* | |
|---|---|---|---|---|---|
| Peak no. | Compound | Male | Female | Male | Female |
| 1 | Pentan-2-one | + | + | | |
| | 3-Hydroxy-3-methylbutan-2-one | | | ++ | ++ |
| 2 | 6-Methylhept-5-en-2-one | + | + | ++ | ++ |
| 3 | *E*, *E*-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane | + | + | | |
| 4 | *E*, *E*-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane | ++ | ++ | | |
| 5 | 2-Methyl-6-pentyl-3,4-dihydro-2H-pyran | | ++ | | |
| 6 | *Z*, *E*-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane | +++ | +++ | | |
| 7 | Undecan-2-one | + | + | | |
| 8 | Undecan-2-ol | + | + | | |
| | Methyl oleate | +++ | +++ | | |

[a]Key: + = minor component, ++ = medium component, +++ = major component.

TABLE 2. RESPONSE OF MALE PARASITOIDS TO LIVE FEMALES, EXCISED HEADS, AND MANDIBULAR SECRETIONS OF FEMALE PARASITOIDS[a]

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Excised heads | | Live females | | Mandibular secretion | |
| | *R. persuasoria* | *M. nortoni* | *R. persuasoria* | *M. nortoni* | *R. persuasoria* | *M. nortoni* |
| Male *R. persuasoria* | 14 | 1 | 39 | 8 | 78 | 16 |
| Male *M. nortoni* | 1 | 9 | 25 | 59 | 50 | 156 |
| $\chi^2$ | 15.4 | | 34.16 | | 89.77 | |
| Significance | $P < 0.001$ | | $P < 0.001$ | | $P < 0.001$ | |

[a]Key response was the number of visits made by males, from 25 of each species, to the presentation point over two minutes.

## DISCUSSION

Males of both parasitoid species aggregate at potential emergence sites by a common stimulus of fungal origin (Madden, 1968; Madden and Coutts, 1979). Male *M. nortoni*, the larger of the two species, excludes *R. persuasoria* where they compete for a common site, and this exclusion is complemented by the release of repellent mandibular secretions from a compact aggregation. *R. persuasoria* males will then stand outside the aggregation and may reform if the emerging insect results in the dispersal of *M. nortoni.*

It has been demonstrated that the mandibular glands produce distinctly different compounds which confer species identification. In *M. nortoni* these compounds include a number of closely related spiroacetals, while in *R. persuasoria* the species-specific compound would appear to be 3-hydroxy-3-methylbutan-2-one. 6-Methylhept-5-en-2-one may act as a repellent and alarm pheromone. After the first report of this compound in ants (Cavill and Ford, 1953), it has been confirmed to be associated with alarm and defense in numerous species of ants (Regnier and Law, 1968; Blum, 1978), as well as in secretions of *Andrena* bees (Tengo and Bergstrom, 1976), other parasitic bees (Hefetz et al., 1982) and some beetles, such as the Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins) (Ryker et al., 1979). The species-specific compounds may act to synergize the repellent effect of the secretion while masking or potentiating its effect on the precopulatory activity of conspecifics.

Compound 4 was first found together with the *Z,E* isomer in three vespine wasp species (Francke et al., 1978), while compounds 3 and 6 were first reported in the mandibular secretions of *Andrena* bees (Francke et al., 1980a) together with the two former spiroacetals. The spiroacetals represent a class of compounds increasingly being identified as having pheromone activity. The first isolation of them from insects would appear to be 'chalcogran' (2-ethyl-1,6-dioxaspiro[4.4]nonane), an aggregation pheromone of the beetle, *Pityogenes chalcographus* (Francke et al., 1977). Cyclic acetals such as brevicomin (Silverstein et al., 1968) have, of course, been known for some time as insect pheromones, again principally as attraction or aggregation pheromones. Since the first reported spiroacetal, a range of these compounds has been found in bees (Francke et al., 1981; Tengö et al., 1982; Bergström et al., 1982), wasps (Francke et al., 1979) and the olive fly (Baker et al., 1980). The ring systems include dioxaspiro[4.4]nonanes, -[4.5]decanes, -[5.5]undecanes, -[4.6]undecanes, and -[5.6]dodecanes. The alkyl substituents range from methyl to butyl, with over 20 skeletons identified, excluding stereoisomers. These are almost always based on an odd carbon number and unbranched skeletons, as are the three found in *M. nortoni*. In these reports and the other references to spiroacetals, the function assigned has generally been that of attraction to members of the same species.

Undecan-2-one and undecan-2-ol, which are biosynthetically related to the spiroacetals found in *M. nortoni* were also found in association with the spiroacetals in *Andrena* bees (Bergström et al., 1982). These two compounds are widespread among the Hymenoptera. There appear to be few reports of normal fatty acid methyl esters appearing in insects generally. Methyl oleate was found along with the methyl esters of other fatty acids in extracts of the Argentine ant, *Iridomyrmex humilis* (Cavill et al., 1980). 3-Hydroxy-3-methylbutan-2-one has only been isolated once before from an insect source (Francke et al., 1974), as an attractant for the ambrosia beetle, *Xyloterus domesticus*. Pentan-2-one has not been commonly found in insects, but has been detected in cockroach secretions (Brossut, 1978), beetles (Moore and Brown, 1979), and bumblebees (Cederberg, 1977), again in mandibular secretions.

Although no mixed species aggregations were observed in the present study, the phenomenon is common among *Megarhyssa* species (Heatwole et al., 1963; Crankshaw and Matthews, 1981) and could be due to the presence of similar spiroacetals in other members of the genus, with final recognition only occurring when the emergent insect releases its secretion. In contrast, *R. persuasoria* is predominantly allopatric with respect to other *Rhyssa* species (Kirk, 1975).

Male aggregations have also been observed in the ichneumonid *Certonotus tasmaniensis* (Turner) which is an indigenous species which has adapted to attack *S. noctilio* in radiata pine. This species also has a characteristic odor. Cephalic secretions are not confined solely to the Ichneumonidae, as myrtenol and methyl oleate were found to be the major volatiles in the mandibular secretion of *Schletterarius cinctipes* (Cresson) of the family Stephanidae. (Davies and Madden, unpublished results).

It is, therefore, apparent that mandibular secretions occur within the parasitic Hymenoptera, acting to facilitate species recognition in mating behavior and the possible exclusion of competing species during oviposition.

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# ESTIMATING MAXIMUM HORIZONTAL AREA OF PHEROMONE PLUMES

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**Abstract**—Graphs and simple Gaussian plume equations are presented for estimating the maximum horizontal area within a pheromone plume. In its simplest form the area, $A_R$, for a given scaling factor, $R = Q/(Ku)$, is $A_R = A_1 R^{\beta}$, where $Q$ is the release rate, $K$ is a specified concentration threshold, $u$ is the wind velocity, and $\beta$ is an atmospheric stability index. Estimates of $A_1$ and $\beta$ are given for several atmospheric stability typing schemes applicable to field and forest habitats.

**Key Words**—Atmospheric diffusion, chemical communication, Gaussian plume model, isopleth area, pheromone, plume, plume area.

## INTRODUCTION

In the past decade much attention has been directed towards theoretical and empirical studies of pheromone dispersal within the atmosphere. Among these are theoretical studies which examine mating and trapping within insect populations (e.g., Hartstack et al., 1976; McClendon et al., 1976; Geiszler et al., 1980) and empirical studies designed to determine the active space or communication distance associated with a pheromone plume (e.g., Farkas and Shorey,

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1974; Aylor et al., 1976; Nakamur and Kawasaki, 1977; David et al., 1982; Baker and Kuenen, 1982). These works range from qualitative discussions of the characteristics of a plume to detailed mathematical analyses of plume structure, and all include attempts to describe the dimensions and structure of a plume. Many of these investigations assumed that the plume was described by its time-averaged concentration profile and were concerned with the magnitude of the horizontal area (i.e., area within a concentration isopleth in the horizontal plane) of the plume at the source height (e.g., Hartstack et al., 1976; Geiszler et al., 1980). However, none of them present solutions that are both simple and applicable to a wide range of environmental conditions.

The goal of this paper is to present simple equations based on the Gaussian plume model for estimating the horizontal plume area at the source height which are applicable to field and forest conditions. The only information required is knowledge of (1) the atmospheric stability, (2) the pheromone release rate, (3) the concentration threshold defining the boundary of the plume, and (4) the mean wind velocity.

The first equation is developed from a slight modification of the elliptical approximation approach used by Nishiwaka (1959) and Hartstack et al. (1976). The second equation is derived directly from the properties of the elliptical approach and is much less mathematically complex. A third set are special cases of the first and second equations applicable primarily to short plumes. All of the equations are parameterized to be consistent with two widely used atmospheric turbulence typing schemes.

## METHODS AND MATERIALS

*Plume Model.* The Gaussian atmospheric diffusion model in the form presented in Fares et al. (1980) was used to describe the pheromone concentration within a plume. This model represents the time-averged concentration profile of pheromone within a fully developed plume. The Gaussian plume model is mathematically simple and has achieved reasonable success (Slade, 1968; Hanna et al., 1982) in explaining a wide variety of observed plume concentration patterns.

The model assumes that at each point downwind the pheromone is distributed around a centerline in both the horizontal and vertical directions according to normal distributions. The standard deviations for each direction increase with distance downwind and the instability of the air. Unstable air exhibits a large amount of turbulence which causes rapid dilution of the pheromone.

To implement the plume model, a Cartesian coordinate system is used to represent the spatial arrangement of the plume and its source. The positive $x$ axis represents the downwind distance from the source. The $y$ axis represents

the horizontal dimension, and the $z$ axis represents the vertical dimension. The pheromone source is assumed to be at the point (0, 0, H) in the coordinate system.

Since a proportion of the pheromone that contacts the ground may be reflected or carried back into the air, the model assumes that there is an identical virtual source at the point (0, 0, −H) below the plane representing the ground. The aboveground portion of this plume is multiplied by the proportion being reflected (i.e., not adsorbed) and is added to the aboveground plume.

The Gaussian plume model for the concentration at the point $(x, y, z)$ downwind for a source at height $H$ with the proportion $\alpha$ being reflected from the ground can be written as

$$C(x, y, z; H) = \frac{Q}{2\pi\sigma_y(x)\,\sigma_z(x)\,u} \exp\left[-\frac{1}{2}\left(\frac{y}{\sigma_y(x)}\right)^2\right] \cdot \left\{\exp\left[-\frac{1}{2}\left(\frac{z-H}{\sigma_z(x)}\right)^2\right] + \alpha \exp\left[-\frac{1}{2}\left(\frac{z+H}{\sigma_z(x)}\right)^2\right]\right\} \quad (1)$$

where $Q$ is the emission rate of the pheromone source, $u$ is the average wind velocity, and $\sigma_y(x)$ and $\sigma_z(x)$ are the standard deviations for the pheromone concentration in the horizontal and vertical directions, respectively (Fares et al., 1980). Collectively, $\sigma_y(x)$ and $\sigma_z(x)$ are called the "dispersion parameters," since they alone determine the rate at which the plume spreads out downwind. The units associated with each parameter depend upon the system under study, but must be consistent throughout the equation.

The plume, specified by the concentration levels downwind (equation 1), is defined as the set of all points at which the concentration is greater than or equal to a threshold level $K$. This implies that the boundaries of the plume occur at $C(x, y, z; H) = K$. In most applications to date (e.g., Hartstack et al., 1976), $K$ has been set to the behavioral response threshold of the receiver. Further, since the mode of the vertical concentration distribution is at the source height, the greatest area on a horizontal plane through the plume also occurs at the source height.

Wall et al. (1981) demonstrated both chemically and electrophysiologically that significant levels of ($E$, $E$)-8,0-dodecadien-1-yl-acetate, the sex attractant of the pea moth, *Cydia nigricana*, were adsorbed to wheat leaves when released from attractant-baited traps. Because incorporating pheromone reflection for elevated sources does not allow the area to be calculated using the technique to be presented and significant adsorption of pheromone has been demonstrated, we assume that there is no reflection of pheromone from the ground for elevated sources. We do present simple adjustments to obtain the areas for ground-level sources. Accordingly, the boundaries at the source height, whether elevated or at ground level, for a plume without reflection (i.e., $z = H$ and $\alpha = 0$) are

constrained to

$$K = \frac{Q}{2\pi\sigma_y(x)\ \sigma_z(x)\ u} \exp\left[-\frac{1}{2}\left(\frac{y}{\sigma_y(x)}\right)^2\right] \quad (2)$$

The boundaries for a ground-level plume with the proportion $\alpha$ being reflected are constrained to equation 2 with $Q^* = (1 + \alpha)Q$ substituted for $Q$.

*Dispersion Parameters.* The standard deviations associated with the horizontal and vertical plume concentration distributions depend upon the degree of air stability. Turbulent eddies, caused by mechanical obstructions and convection, carry pheromone away from the path associated with the mean direction of the wind. The mean wind speed and eddy size determine the downwind pheromone dispersal rate. Accordingly, the dispersion parameters will differ for different terrain types and vegetation conditions. Theoretical and empirical studies (Slade, 1968; Hanna et al., 1982) of atmospheric diffusion show that at short distances downwind ($x <$ ca. 500 m) the plume concentration standard deviations are approximately equal to the wind direction standard deviation in radians in the appropriate plane ($y$ or $z$) multiplied by the distance downwind. At larger distances the standard deviations become proportional to the square root of the downwind distance.

The dispersion parameters increase with increasing sampling time (i.e., time over which the concentration is averaged) during their measurement. This is a result of the effects of larger eddies becoming measurable as the sampling interval increases. Because these eddies carry the pheromone greater distances across or above and below the centerline, the average concentration at any point decreases. This decrease is expressed as an increase in the magnitude of the dispersion parameters. Although this occurs in both directions, the primary influence of sampling time is on the horizontal coefficient in the first few hundred feet from the ground (Hanna et al., 1982). Crude estimates (Hanna et al., 1982) for the horizontal dispersion parameter, $\sigma_y(x)$, for a sampling time $T$ can be obtained from the coefficient $\sigma_y^*(x)$ measured over a sampling time of $T^*$ using the relationship

$$\sigma_y(x) = \sigma_y^*(x)\ (T/T^*)^q \quad (3)$$

where $q = 0.2$ for 3 min $< T <$ 1 hr and $q = 0.25$–$0.30$ for 1 hr $< T <$ 100 hr.

A variety of schemes have been used to categorize atmospheric turbulence into stability classes and to describe dispersion downwind within a class (Gifford, 1976). A simple scheme that is widely used because it has produced satisfactory results in most cases (Hanna et al., 1982) was developed by Pasquill (1961). He divided atmospheric stability into six categories according to observed wind speed, cloud cover, and insolation levels. These categories and the conditions with which they are associated in open field and forest habitats are presented in Table 1. Pasquill also presented graphs describing the lateral and

TABLE 1. PASQUILL[a] AND FARES STABILITY CATEGORIES ASSOCIATED WITH HABITAT TYPES AND WEATHER CONDITIONS[b]

Low crops and open terrain:

| Surface wind speed (m/sec) | Daytime insolation | | | Nighttime conditions | |
|---|---|---|---|---|---|
| | Strong | Moderate | Slight | Thin overcast or $\geq \frac{4}{8}$ cloudiness[c] | $\leq \frac{3}{8}$ cloudiness |
| <2 | A | A–B | B | F[d] | F[d] |
| 2 | A–B | B | C | E | F |
| 4 | B | B–C | C | D | E |
| 6 | C | C–D | D | D | D |
| >6 | C | D | D | D | D |

Open Forests:

| Category | Temperature profile[e] | Corresponding Pasquill categories | Identifier[f] |
|---|---|---|---|
| Inversion | $\Delta T > 1.5$ | F | I |
| Intermediate | $0 \leq \Delta T \leq 1.5$ | D, E | J |
| Buoyant | $\Delta T < 0$ | A, B, C | |
| (a) upward | | | K |
| (b) downward | | | I |

[a]Pasquill stability categories [$\sigma_\theta$ = wind direction SD]: A = Extremely unstable conditons [$\sigma_\theta$ = 25°], B = Moderately unstable conditions [$\sigma_\theta$ = 20°], C = Slightly unstable conditions [$\sigma_\theta$ = 15°], D = Neutral conditons (applicable to heavy overcast day or night) [$\sigma_\theta$ = 10°], E = Slightly stable conditions [$\sigma_\theta$ = 5°], F = Moderately stable conditions [$\sigma_\theta$ = 2.5°].
[b]After Slade (1968) and Fares et al. (1980).
[c]The degree of cloudiness is defined as that fraction of the sky above the local apparent horizon that is covered by clouds.
[d]F suggested, however, A–F can occur in these conditions. Selection should be based on $\sigma_\theta$.
[e]$\Delta T$ is defined as the temperature change in °C between 0.5 and 8.0 m from the floor of the canopy.
[f]Arbitrarily selected letters used to identify the categories for which Fares et al. (1980) supplied parameter estimates.

vertical spreading of a plume with distance downwind in terms of the 10% points of plume concentration relative to the mean centerline value for open, level terrain. Gifford (1961) expressed these graphs in terms of $\sigma_y(x)$ and $\sigma_z(x)$ to produce the set of widely used curves now called the Pasquill–Gifford (PG) curves. A second set of widely used curves, also presented in equation form, was developed by Briggs (1973). These curves are similar to the PG curves, but they incorporate information from other studies. Although the PG curves and Briggs formulas are two of the most widely used dispersion typing schemes, the equa-

tions describing these curves over their entire range unfortunately do not allow the development of simple equations for plume area.

In many investigations the observed plume standard deviations have been described using simple power functions (e.g., Cramer et al., 1958; Fares et al., 1980). Accordingly, the equations for the dispersion parameters for a given stability class are

$$\sigma_y(x) = ax^b \tag{4}$$

and

$$\sigma_z(x) = cx^d \tag{5}$$

As we will show, these formulas allow the development of simple area equations, but their general limitation is that no single power function can fit diffusion data over all downwind distance ranges (Gifford, 1976). However, Tadmor and Gur (1969) demonstrated that power functions approximated the PG curves well for downwind distance less than 5 km. For distances less than 500 m Briggs formulas are accurately approximated by simple lines (i.e., $b = d = 1$). In this case $a$ and $c$ are approximately equal to the wind direction standard deviations in the horizontal and vertical directions, respectively. Since most pheromone plumes are less than 500 m in length, power function approximations to the PG and Briggs curves will allow the accurate calculation of plume areas associated with these two popular stability classification schemes.

Equations 4 and 5 will be used to describe the dispersion parameters in this study. Estimates of $a$, $b$, $c$, and $d$ for the open field conditions associated with the PG curves for each stability category were taken from Eimutis and Konicek (1972) and are presented in Table 2. The values for $\sigma_z(x)$ are for downwind distances less than 100 m. These values were selected here for $\sigma_z(x)$ since a majority of the events in pheromone communication occur within 100 m of the source. For estimates of Briggs' formulas, $a$ and $c$ were assigned the value of the coefficient in the numerator of his equations, and $b$ and $d$ were set equal to one (Table 2). The maximum relative error at 500 m over all stability classes incurred by using these estimates is 2.5% for $\sigma_y(x)$ and 32.3% for $\sigma_z(x)$. Values of $a$, $b$, $c$, and $d$ for open, pine forest conditions were taken from Table 6 in Fares et al. (1980) (Table 2). As presented here, equation 5 will underestimate $\sigma_z(x)$ for inversion conditions is forests, since it does not include the height of the inversion layer.

The Pasquill and Briggs estimates are applicable to 10-min sampling times and the values for the Fares estimates are based on 15-min sampling times. The horizontal parameters for other sampling periods can be estimated using equation 3. These parameter sets are based on data collected in open, level terrain and open pine forest conditions, and dispersion parameter constants for other habitat types could be different. Further, it must be noted that none of the

TABLE 2. POWER FUNCTION CONSTANTS DEFINING DISPERSION PARAMETERS IN GAUSSIAN PLUME MODEL

| Stability category[a] | Power function constants[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Horizontal | | Vertical | | | | |
| | a | b | c | d | $F^{c,d}$ | $A_1{}^{c,d}$ | $\beta$ |
| A (P) | 0.37 | 0.90 | 0.19 | 0.94 | 0.74851 | 1.11688 | 1.03261 |
| (B) | 0.22 | 1 | 0.20 | 1 | 0.73057 | 0.99736 | 1 |
| B (P) | 0.28 | 0.90 | 0.16 | 0.92 | 0.74851 | 1.35798 | 1.04396 |
| (B) | 0.16 | 1 | 0.12 | 1 | 0.73057 | 1.66226 | 1 |
| C (P) | 0.21 | 0.90 | 0.12 | 0.90 | 0.74851 | 1.88670 | 1.05556 |
| (B) | 0.11 | 1 | 0.08 | 1 | 0.73057 | 2.49339 | 1 |
| D (P) | 0.15 | 0.90 | 0.08 | 0.88 | 0.74851 | 3.02401 | 1.06742 |
| (B) | 0.08 | 1 | 0.06 | 1 | 0.73057 | 3.32452 | 1 |
| E (P) | 0.10 | 0.90 | 0.06 | 0.87 | 0.74851 | 4.29713 | 1.07345 |
| (B) | 0.06 | 1 | 0.03 | 1 | 0.73057 | 6.64904 | 1 |
| F (P) | 0.07 | 0.90 | 0.05 | 0.81 | 0.74851 | 6.08839 | 1.11111 |
| (B) | 0.04 | 1 | 0.016 | 1 | 0.73057 | 12.46694 | 1 |
| I | 0.007 | 1.4 | 1.51 | 0.2 | 0.65759 | 0.34878 | 1.50000 |
| J | 0.007 | 1.4 | 0.99 | 0.42 | 0.65759 | 0.39696 | 1.31868 |
| K | 0.007 | 1.4 | 0.47 | 0.6 | 0.65759 | 0.70135 | 1.20000 |

[a]B = Approximation to Briggs (1973) formulas. P = approximation to the Pasquill-Gifford curves.

[b]Pasquill coefficients extracted from Tables 1 and 3 in Eimutis and Konicek (1972). Numerator coefficient for Briggs formulas taken from Table 4.5 in Hanna et al. (1982). Forest coefficients taken from Table 6 in Fares et al. (1980).

[c]Based on Simpson's rule approximation of the actual areas using 10,000 subintervals and double-precision arithmetic.

[d]Values represent the averages for $R$ equaling 1, $10^2$, $10^4$, $10^6$, and $10^8$. The actual deviation between any two observed values within a stability category was $<10^{-6}$ in every case.

atmospheric diffusion data from which these dispersion parameters were estimated were directly concerned with pheromone communication systems. They are only used here due to lack of alternatives.

*Derivation of Plume Area Equations.* Nishiwaka (1959) observed that the area of an ellipse closely approximated the plume area when the plume's length is used as the ellipse's length and its maximum width is used as the ellipse's maximum width. Hartstack et al. (1976) also used this approach to estimate the plume area. The length (Length), width as a function of distance downwind [$W(x)$], distance downwind at which the width is greatest ($XW_{max}$), and the

TABLE 3. EQUATIONS DESCRIBING DIMENSIONS OF TIME-AVERAGE PLUME (AREA WITHIN CONCENTRATION ISOPLETH) AT SOURCE HEIGHT FOR GAUSSIAN PLUME MODEL [a]

| Dimension | Equation [b] | No. |
|---|---|---|
| Length | $\text{Length} = \left(\frac{R}{2\pi ac}\right)^{1/(b+d)}$ | (T3.1) |
| Width at downwind distance $x$ | $W(x) = \{8a^2x^{2b} \log_e [R/(2\pi acx^{b+d})]\}^{1/2}$ for $0 < x \leqslant \text{Length}$ | (T3.2) |
| Downwind distance of maximum width | $XW_{\max} = (\text{Length}) \exp[-1/(2b)]$ | (T3.3) |
| Maximum width | $\text{Width} = 2a \exp[-1/2] [(b+d)/b]^{1/2} (\text{Length})^b$ | (T3.4) |
| Area | $A_R = (F) (\text{Length}) (\text{Width})$ | (T3.5) |
| | $= A_1 R^{\beta}$ | (T3.6) |
| | where | |
| | $F = (\pi/4) \exp\{-0.0847b + 0.0044b^2$ | |
| | $- 0.1602 \log_e(b) - 0.1320 [\log_e(b)]^2\}$ | (T3.7)[c] |
| | $\beta = (b+1)/(b+d)$ | (T3.8) |

[a]Dispersion parameters: $\sigma_y(x) = ax^b$ and $\sigma_z(x) = cx^d$.
[b]$R = Q/(Ku)$ for elevated or ground-level sources with complete pheromone adsorption to the ground. $R = (1 + \alpha)\, Q/(Ku)$ for ground-level sources which reflect the proportion $\alpha$ of the pheromone contacting the ground back into the air.
[c]$F$ is accurate to within $0.01\pi/4$ over the range $0.1 \leqslant b \leqslant 10.0$.

maximum width (Width) are given in Table 3 by equations T3.1–T3.4, respectively. These equations can all be derived directly from equation 2 and are expressed as functions of the scaling factor, $R = Q/(Ku)$. This factor represents an adjustment of the ratio ($Q/K$) of the release rate to the response threshold identified by Bossert and Wilson (1963) as the primary measure describing olfactory communication. Because the wind speed determines the time which a volume of air has to pick up the pheromone being released as it passes the source, there will be less pheromone in the volume of air at higher wind speeds than at lower speeds. Accordingly, $Q/u$ represents the apparent release rate when monitoring the concentration in a volume of air, which could be quite different from the biologically meaningful pheromone flux rate (see Elkinton and Cardé, 1984), and $R$ could be thought of as the effective communication ratio.

Using the dispersion parameter constants in Table 2, plume area estimates calculated using the area of an ellipse [i.e., $\pi$(Length) (Width)/4] were compared to the actual plume areas. The maximum absolute relative error incurred by using the area of an ellipse was 4.7% for Pasquill's categories and 16.3% for

Fares' forest stability categories. This error was similar to that observed by Nishiwaka (1959). Actual plume areas were obtained here and for subsequent parameter estimations by integrating equation T3.2 using Simpson's rule with 10,000 subintervals and double-precision arithmetic on a PRIME 400® computer. In determining the error associated with approximating plume area by the area of an ellipse, it was observed that for a given stability category the relative error was constant over all values of $R$. A small variation of the order of $10^{-6}$–$10^{-8}$ was observed, but this was assumed to be due to the truncation and round-off error associated with the Simpson's rule estimation. This belief is based on the observation that the variation consistently decreased as the number of integration subintervals used increased. This implied that precise values of the plume area at the source height for a given set of dispersion parameters and value of $R$ could be obtained by multiplying the area of the ellipse by a constant correction factor, $f$. Sensitivity analysis showed that the correction factor $f$ depended solely upon the value of $b$.

Using these observations, formulas for the plume area base on correcting the area of the equivalent ellipse were derived through simple algebraic substitution and simplification. An approximation for the correction factor as a function of $b$ was obtained using multiple linear regression on transformed data using exact values of $f$ calculated over a range of $b$ between 0.1 and 10.0.

*Approximation Accuracy.* To compare the agreement of these approximations to the true areas associated with plumes defined by the actual PG and Briggs dispersion parameters, the correct areas were plotted along with the approximations in the same graph. Actual areas calculated with PG curves for a ground-level source with complete reflection were determined graphically by Hilsmeier and Gifford (1962) for $R$ greater than 100. These values were adjusted to correspond to plumes without reflection using equation 11 (see below). Numerical integration, using Simpson's rule with 2000 subintervals, was used to obtain the actual areas associated with Briggs formulas as the dispersion parameters. Because the forest diffusion parameters were given as power functions, the estimates are exact for the intended model in all cases except the inversion conditions. Excluding inversion conditions, the only error is the discrepancy between the model and a real plume. Under inversion conditions, the approximation will increasingly overestimate the plume area as the height of the inversion layer increases.

## RESULTS

*Plume Area Equations.* Based on the analytical observations in the methods section, very precise areas within a concentration isopleth at the source height for a plume without reflection from the ground can be calculated as

$$A_R = (F)(\text{Length})(\text{Width}) \tag{6}$$

where $A_R$ is the area for a given $R$, $F = f\pi/4$ is the modified correction factor, and Length and Width are equations T3.1 and T3.4, respectively, in Table 3. Exact values of $F$ for each set of dispersion parameters are given in Table 2. The approximation of $F$ as a function of $b$ is given in Table 3 as equation T3.7. This function predicts within $0.01(\pi/4)$ of the true value over the range $0 \leq b \leq 10.0$.

Substitution of the equations for Length and Width into equation 6 allows $A_R$ to be calculated directly from the dispersion parameter constants, $F$ and $R$, as

$$A_R = 2Fa \exp\,[-1/2][(b + d)/b]^{1/2}\left(\frac{R}{2\pi ac}\right)^{(b+1)/(b+d)} \tag{7}$$

A simpler equation than 6 or 7 can be obtained by factoring $R$ out of equation 7 and recognizing that the value of the terms not including $R$ equals $A_1$, i.e., the area of a plume having $R = 1$. Accordingly, the plume area can be calculated by a simple power function as

$$A_R = A_1 R^{\beta} \tag{8}$$

where $\beta = (b + 1)/(b + d)$. Equation 8 states that within a stability category the area for any plume can be obtained by scaling the area of a plume with $R = 1$. Thus, under the Gaussian plume model with power function dispersion parameters, the accuracy of $A_R$ is solely dependent upon the accuracy of $A_1$. Exact values of $A_1$ are presented for each stability category in Table 2.

In cases where the exponents of the dispersion parameters equal one (i.e., $b = d = 1$), as with the estimates to Briggs formulas or short plumes where the wind direction standard deviations can be used for $a$ and $c$, equations 7 and 8 simplify greatly. They become, respectively,

$$A_R = FR/[c\pi(0.5 \exp\,[1])^{1/2}] \tag{9}$$

$$A_R = A_1 R \tag{10}$$

Both equations are simple lines passing through the origin with slope $A_1$ when $A_R$ is plotted against $R$. This simplicity allows rapid calculation of accurate estimates of plume area when the short plume estimates of Briggs formulas are used.

To calculate the plume area associated with a ground-level source for which the proportion $\alpha$ of the phermone contacting the ground is reflected back into the atmosphere,

$$R^* = (1 + \alpha)R \tag{11}$$

is substituted for $R$ into equations 6 through 10. Unfortunately, this substitution does not hold for elevated sources. To illustrate the use of equation 11 for ground-level sources, the effect of adsorption on the plume area associated with the dermestid *Trogoderma glabrum* will be examined. Shapas and Burkholder (1978) estimated the average peak release rate of 14-methyl-8-hexadecenal by females as $Q = 3.2 \times 10^{-2}$ ng/sec and the threshold for response by 50% of the males as 1 ng/m$^3$. At a wind velocity of 0.50 m/sec, $R = 3.2 \times 10^{-2}/[(0.50)(1)] = 0.0640$ m$^2$. If this occurred in the open under moderate-to-slight insolation (Pasquill category B), the area of a female's plume using Briggs coefficients (which may be poor estimates for such small plumes) and equation 10 would be $A_{0.0640} = (1.66226)(0.0640) = 0.106$ m$^2$, assuming that all pheromone contacting the ground is adsorbed. However, if only 25% of the pheromone is adsorbed, $R^* = (1 + 0.75)(0.0640) = 0.1120$ m$^2$ and the resulting plume area is $A_{0.1120} = 0.186$ m$^2$. Although this is not a dramatic increase, it does illustrate the importance of adsorption on plume area.

*Approximation Accuracy.* Graphs of the plume area associated with the PG curves for each stability category plotted against R, as predicted by equation 8, are presented as the broken lines in Figure 1. The solid lines in the graph are the areas calculated by Hilsmeier and Gifford adjusted to remove reflection. The numbers in parentheses are the lengths of each plume at $R$ equal to $10^4$ calculated using equation T3.1. Like the areas, these values demonstrate the importance of turbulence in the rapid dilution of pheromone. The plume for the most stable category, $F$, is almost nine times as long as the plume in the least stable category, $A$. As shown, the approximations based on the dispersion parameter values in Table 2 agree well with the Hilsmeier and Gifford areas even for plumes greater than 100 m in length. The approximation for $F$ deviates the greatest from the Hilsmeier and Gifford areas, and is consistently lower than the Hilsmeier and Gifford predictions. However, the maximum difference in the range graphed is only 25% and should cause little problem in application.

Approximations using equation 10 (broken lines) and numerically integrated areas using Briggs formulas show excellent agreement between the two sets of curves (Figure 2). As before, the only significant deviation between the approximation and the areas actually defined by Briggs formulas occurs in category $F$. At $R = 1$ there is less than 0.5% error, and the error goes from ca. 15% at $R = 1000$ to 50% at $R = 10^4$. Note that the areas associated with Briggs formulas agree well with Hilsmeier's and Gifford's estimates for all stability categories, except $A$. The areas for $A$ associated with Briggs formulas are approximately 40% smaller than Hilsmeier's and Gifford's areas. This is to be expected since Briggs defined $\sigma_z(x)$ for category $A$ to represent more unstable conditions than defined by the PG curves.

For both the PG curves and Briggs formulas the area associated with the most stable category, $F$, is approximately 10 times that of the least stable cate-

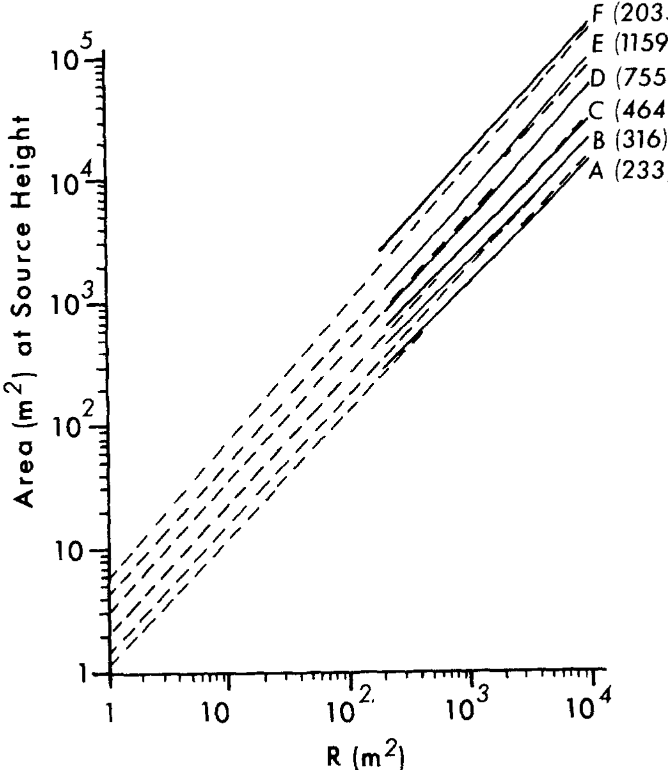


FIG. 1. Areas for plumes without reflection defined by the Plasquill–Gifford diffusion parameters. Solid lines represent the adjusted Hilsmeier–Gifford estimates, and the broken lines are the approximations given by equation 8. Plume lengths for $R = 10^4$ are in parentheses.

gory, *A*. In general the approximations based on the PG curves are smaller than those for Briggs formulas for small *R* and larger than those for large *R*. The greatest deviation occurs between the approximations in category *F*. For $R = 10^4$, the PG area is only ca. 25% greater than the Briggs area approximation. However, at $R = 1$, the Briggs area is twice that predicted for the PG curves. The estimate associated with the PG curves is probably more reliable since the tabled dispersion parameter values are for plumes less than 100 m in length. For both sets of approximations, little error will be accrued in categories *A*, *B*, and *C* when using the approximations for several orders of magnitude above $R = 10^4$. However, for categories *E* and *F* for the PG curves and *D*, *E*, and *F* for the approximations to Briggs areas, the approximations will underestimate the mean plume area for values of $R > 10^4$. Except for very large values of *R* (e.g., $10^6$), this error will be less than an order of magnitude.

Figure 3 contains the areas associated with the dispersion parameters for

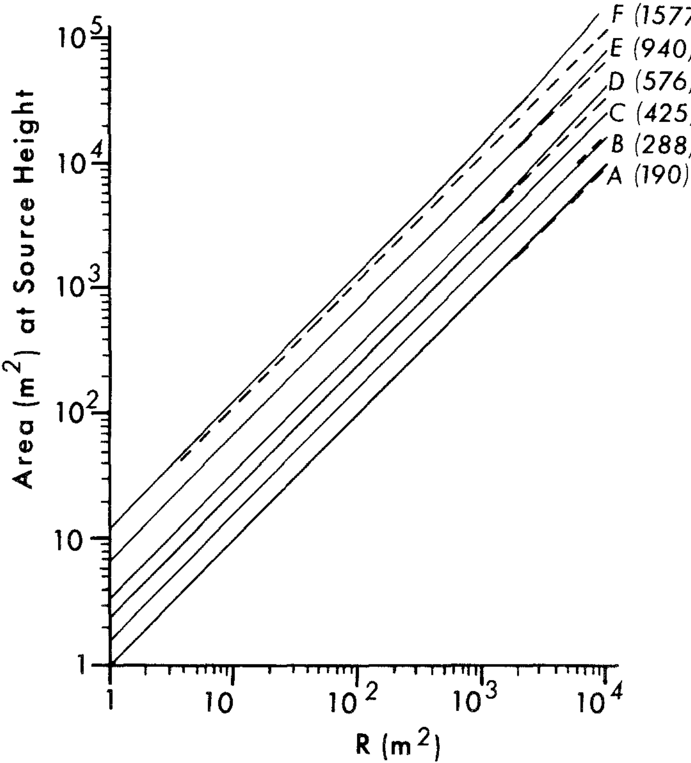


FIG. 2. Areas for plumes without reflection using dispersion parameters defined by Briggs formulas. Solid lines represent the numerically integrated plume model, and the broken-lines are the approximations given by equation 10. Plume lengths for $R = 10^4$ are in parentheses.

forest habitats in Table 2. These lines are exact for the three categories since the dispersion parameters were defined as power functions. For $R < 30$, the areas for all the categories are similar, and categories $J$ and $K$ are similar for the entire range graphed. At $R = 10^4$, the area associated with category $J$ is only twice that of $K$, and category $I$, which is more stable, has an area 10 times that of $K$. In general, the areas associated with the forest habitat differ greatly from those associated with the PG curves and Briggs formulas for open areas, but at $R >$ 1000 the areas of $I$, $J$, and $K$ are similar to $F$, $D$, and $E$, respectively.

## DISCUSSION

To estimate the plume areas for a given habitat and stability category, the areas can be read directly from Figures 1, 2, or 3. Adjustment for pheromone

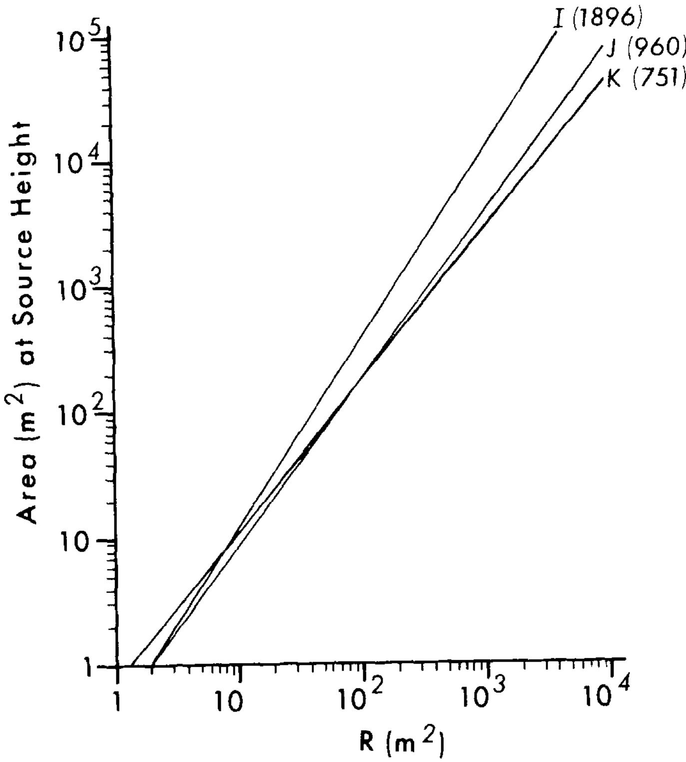


FIG. 3. Areas for plumes without reflection using the forest dispersion parameters defined by Fares et al. Plume lengths for $R = 10^4$ are in parentheses.

reflection off the ground for gound-level sources is achieved by shifting the value of $R$ according to equation 11. However, equations 7 through 10 provide very simple formulas for calculating the areas using the parameters in Table 2. Equation 10 is especially simple as it is a line. Since Briggs formulas are generally accepted as reasonable estimates among workers in atmospheric diffusion (Gifford, 1976, Hanna et al., 1982), this equation should be a useful tool to researchers studying pheromone communication. Further, the equations developed here are immediately applicable to any stability classification scheme in which the dispersion parameters are defined or approximated by power functions. To estimate the areas for a stability class, estimate the correction factor using equation T3.7. The area $A_1$ for $R = 1$ is calculated using equation 7, or equation 9 if $b$ and $d = 1$. The areas associated with a value of $R$ can then be calculated using equation 8, or equation 10 if $d = 1$. Equation 11 can be used to adjust for reflection for ground-level sources.

A significant point in this study is that a power function of $R$ (i.e., eq. 8)

gives the plume area associated with the Gaussian plume model with the dispersion parameters defined as power functions. Elliott (1959) and Elliott and Nickola (1961) demonstrated empirically, using field data, that within a stability class the plume area can be described well by a power function of $R$. It is reassuring that the form of equation 8 is in agreement with previous empirical observations. Slade (1968) presents an unpublished study by Gifford in which he compares Elliott's areas to the Hilsmeier and Gifford curves. In general, the two sets of curves were in fair agreement with one another.

Despite this empirical support, the relevance and limitations of the equations and graphs presented in this paper should be considered when applying these relationships to field situations. They provide quantitative measurements of the attributes of the Gaussian plume model, but are not improvements over the Gaussian model. Discussions of the reliability of the Gaussian plume model can be found in Slade (1968), Fares et al. (1980), and Hanna et al. (1982). The Gaussian plume model is relevant to the meandering plume's average behavior in variable wind directions (David et al., 1982), since each Pasquill stability category can be associated with a specific wind standard deviation range (Slade, 1968). The equations presented in this paper will be useful for estimating the area in the field that, on the average, is exposed to at least a threshold concentration. However, because the plume model represents the long-term average concentration at a given point, the actual concentration at a given instant is likely to be much lower or greater than predicted. This was demonstrated by Aylor et al. (1976), who estimated peak disparlure concentrations 24 times greater than the predicted average at 1.2 m from the source. Accordingly, plumes defined by the Gaussian plume model will generally be wider and shorter due to the averaging than the instantaneous plumes.

A recent field bioassay by Elkinton et al.(1984) lends a further note of caution regarding the use of time-average plume models. They were able to estimate the concentration profile of time-averaged plumes using a grid of male gypsy moths, *Lymantria dispar*, set up downwind of a disparlure source in a woodlot. They observed that the qualitative behavior of the plume models was similar to that of the plumes. However, they found that the predicted average concentrations at locations where wing fanning occurred were often several orders of magnitude lower than wing-fanning response thresholds determined in wind tunnel tests. They found the best agreement using the PG dispersion parameters in which the category was selected using the lateral wind direction standard deviation. But even the "best" agreements implied response thresholds 2–4 orders of magnitude lower than those observed in the wind tunnel. Contrary to expectation, they observed the poorest agreement using the forest dispersion parameters presented in Fares et al. (1980). They did point out that their bioassay tended to overestimate the average concentration, since a brief exposure to a concentration greater than the threshold will produce a response even though

the average concentration at the site is below the threshold. As they note, this is certainly a shortcoming of time-average plume models in general.

A very crude estimate in the error between the area over which a peak concentration greater than the threshold occurred sometime during the sampling interval and the area within the threshold concentration isopleth predicted by the Gaussian plume model can be obtained using the results of Elkinton et al. (1984). The former area would estimate the spatial area actually sampled for males by the plume, while the latter area represents the spatial area in which the average concentration over the interval was greater than the threshold. Elkinton et al. reported that the threshold concentration eliciting a wing-fanning response in 50% of the males in wind tunnel bioassays was $K = 1 \times 10^{-18}$ g/cm$^3$, and the release rate from their source loaded with 100 $\mu$g of disparlure was $Q = 296$ pg/sec in a $u = 1.32$ m/sec wind [$R = Q/(Ku) = 224.2$ m$^2$]. Their figures show that concentration isopleths at the source height based on a threshold of $K^* = 1 \times 10^{-20}$ g/cm$^3$ ($R = 22{,}424$ m$^2$) enclosed most stations were $\geq$50% of the males responded. If we use the dispersion parameters for Pasquill category $B$ and equation 8, assuming that isopleths based on $K^*$ define the bounds of 50% male response, then the area sampled by the plume ($A_{22424} = 1.35798(22424)^{1.04396} = 47{,}300$ m$^2$) is 123 times greater than the predicted area ($A_{224.2} = 386$ m$^2$). This is probably an overestimate, since equation T3.1 would indicate that the time-average plume using $K^*$ would be 493 m long. Elkington et al. only monitored response up to 80 m downwind. It is unlikely that 50% male response would be observed ca. $\frac{1}{2}$ km downwind in a woodlot, since there would be significant adsorption of pheromone to the trees. However, Figures 4 and 5 in Elkinton et al. (1984) indicate that the discrepancy in the areas is large. Assuming that the error estimate is close to the true value, then the discrepancy between the two areas is ca. 10 times greater than the relative difference between the Gaussian plume areas in the least and most stable Pasquil stability categories. This makes it clearly evident that more studies like that of Elkinton et al. are needed to assess the usefulness of the Gaussian plume model parameterized with currently available diffusion parameter estimates. These studies should help to identify where the Gaussian model is appropriate and, ultimately supply investigators with a set of dispersion parameter estimates and adjustment factors that will allow realistic estimates of the area within a concentration isopleth to be obtained.

This paper has presented simple equations and graphs for estimating the area of a pheromone plume based on the Gaussian plume model parameterized using the most commonly used dispersion parameter sets. As illustrated above, these parameter sets may not be adequate and more studies are needed. Although the Gaussian plume model may be inappropriate in purely mechanistic studies of pheromone communication, its qualitative behavior and mathematical simplicity insure its use in quantitative studies until a more precise replacement

is developed. A real advantage associated with equation 8 and especially equation 10 is that they can be easily inserted into mating, trapping, and/or communication disruption models with little addition to the complexity of the models. This should promote the development of simpler models describing the population dynamics associated with pheromone communication, and help to improve our understanding of the quantitative ecology of pheromone communication.

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# ACCESS OF LARGE AND NONVOLATILE MOLECULES TO THE VOMERONASAL ORGAN OF MAMMALS DURING SOCIAL AND FEEDING BEHAVIORS

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**Abstract**—The vomeronasal organ, a chemosensory structure in the nasal cavity, is important in the detection of mammalian chemosignals, many of which are thought to be large molecules having low volatility. We conducted a series of experiments to determine whether nonvolatile molecules enter the vomeronasal organ during a variety of behavioral contexts in five species. We found that a nonvolatile dye entered the vomeronasal organ during investigation of urine from conspecific donors (experiment 1), during investigation of urine from heterospecific donors (experiment 2), during self-grooming (experiment 3), and during social grooming (experiment 4). In other experiments, we determined that nonvolatile molecules entered the vomeronasal organ during consumatory behaviors. Animals that ate a dye-adulterated familiar food had the nonvolatile marker in their vomeronasal organs (experiment 5). Animals that drank either familiar or novel dye-adulterated solutions also had the nonvolatile marker in their vomeronasal organs (experiment 6). In Experiment 7, large (66,000-dalton) fluorescent molecules were mixed with female urine which was then presented to male animals. We observed that the large molecules were transported to the vomeronasal organ. In the final experiment, we determined that mere contact between the snout of a dead animal and the stimulus resulted in transport of nonvolatile substances to the vomeronasal organ. We conclude that the vomeronasal organ, like the olfactory epithelium, is in continuous contact with the environment, but unlike the olfactory epithelium, the sensory receptors of the vomeronasal organ interact with molecules of low volatility, in addition to more volatile odorants.

**Key Words**—Vomeronasal organ, Jacobson's organ, olfaction, feeding, grooming, urine, chemicals, volatility, high molecular weight.



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## INTRODUCTION

The vomeronasal chemosensory system plays an important role in the physiology and behavior of mammals. The primary sensory cells, located in the vomeronasal organ, communicate via the accessory olfactory bulb with regions of the central nervous system that have been implicated in the regulation of reproductive physiology and behavior (Winans and Scalia, 1970; Scalia and Winans, 1975; Krettek and Price, 1978; Kevetter and Winans, 1981a,b; Lehman and Winans, 1982; see also Keverne, 1979; and Wysocki, 1979). Not surprisingly, ablation studies have revealed that the vomeronasal system is intimately involved in many events related to reproduction, including sexual maturation (Kaneko et al., 1980; Sanchez-Criado, 1982), modulation of female cyclicity (Johns et al., 1978; Reynolds and Keverne, 1979), pregnancy block (Bellringer et al., 1980), modulation of hormone levels (Wysocki et al., 1983; Coquelin et al., 1984), maternal behavior (Fleming et al., 1979), chemosensory investigation (Beauchamp et al., 1982), aggression (Bean, 1982b; Clancy et al., 1984a), courtship (Bean, 1982a; Wysocki et al., 1982; Clancy et al., 1984a), and mating (Powers and Winans, 1975; Winans and Powers, 1977; Lehman et al., 1980; Meredith et al., 1980; Lehman and Winans, 1982; Clancy et al., 1984a).

Chemical studies have implicated large molecules with low volatility in many of these physiological and behavioral events (see contributions in Müller-Schwarze and Silverstein, 1980, 1983). The vomeronasal organ may play a significant role in the detection of these compounds in part because of its location, near the opening of the nose, and its anatomical relationships with the oral cavity and nasopalatine duct. The observation that (1) a nonvolatile dye readily reached the vomeronasal organ of guinea pigs during investigation of dyed urine (Wysocki et al., 1980a,b), and (2) male hamsters lacking their vomeronasal organs do not respond to the high-molecular-weight components of hamster vaginal discharge are consistent with this view. Similar observations have been reported after the presentation of dyed female goat urine to male goats (Ladewig and Hart, 1980) and contact between the noses of various prosimians and radio-opaque fluids (Schilling, 1980).

No evidence regarding the role of the mammalian vomeronasal organ in nutrient selection is available, although it may be critical in the maintenance of feeding in some reptilians (Kubie and Halpern, 1976). Results from tests of two guinea pigs suggested that samples of ingested compounds also may be transported to the vomeronasal organ; after two guinea pigs consumed rhodamine-adulterated water, the dye was observed in one of the vomeronasal organs (Wysocki et al., 1980a).

In a series of experiments we determined that rhodamine entered the vomeronasal organ during a variety of contexts, including feeding and social behaviors. We also determined that transport of the nonvolatile dye to the vomeronasal organ occurred in a variety of rodent species not previously assessed.

## METHODS AND MATERIALS

Throughout the experiments, we monitored the access of a nonvolatile fluorochrome (rhodamine hydrochloride) to the vomeronasal organ. The dye was placed in various media that the animals were allowed to contact. Subsequent to interacting with the stimulus, animals were immediately sacrificed and their vomeronasal organs were removed, frozen, and sectioned in a freezing microtome. Sections were mounted from the knife onto glass slides and viewed with a Zeiss microscope equipped with epifluorescence. The excitation and emission filters were selected for viewing rhodamine fluorescence. In the absence of rhodamine, no endogenous fluorescence was observed in vomeronasal organ tissue when viewed through rhodamine-specific filters.

We first examined the possibility that rhodamine was transported to the vomeronasal organ during dissection rather than following the animal's contact with the dyed stimulus. Pine voles (*Microtus pinetorum*) and meadow voles (*M. pennsylvanicus*) were sacrificed prior to any contact with rhodamine. The dye (1% w/v in distilled water) was then painted on either the cheek, mouth, vomeronasal organ capsule caudal to its ostium, or the septal organ (another sensory structure located in the nose). After painting, the animal's vomeronasal organ was removed, frozen, and sectioned. Sections were viewed and the presence of rhodamine was noted. Rhodamine was not detected in the vomeronasal organ in any of the conditions. Thus, we conclude that dissection and sectioning of tissue did not result in the artifactual presence of rhodamine in the vomeronasal organ.

### *Experiment 1*

Guinea pigs were initially used in our laboratory to demonstrate that a nonvolatile dye could reach receptors in the vomeronasal organ during or following contact with dyed urine (Wysocki et al., 1980a). Urinary cues also are significant in the chemical communication system of mice and the vomeronasal organ is a critical afferent component (Wysocki et al., 1982). In this experiment, we determined whether dyed urines from mice (*Mus domesticus*) reached receptors of the vomeronasal organ of other mice subsequent to contact with urine.

Forty individually housed adult male DBA/2J mice were used as subjects. Half of the subjects had lived in all-male groups since weaning, and the other half had brief exposures as adults with adult male and female mice. Urine donors were as follows: castrated adult male mice, intact adult male mice, hypophysectomized female mice, and intact female mice. These donors were chosen based upon previous research results indicating differential responsiveness by male mice to the urines of these donors (Nyby et al., 1979). Urine was obtained from donors that were housed overnight in metabolic cages in groups of 3–5 mice. Each subject was exposed to a single urine type. The total number

of experienced and inexperienced males receiving each urine type was balanced, resulting in five animals in each cell.

On the test day, males were exposed to a cotton-tipped surgical swab that was dipped into urine dyed with rhodamine (1% w/v). Each swab was then secured to a phonographic pick-up cartridge. The electrical output from the cartridge was amplified and visualized on an analog chart recorder. Contacts between mouse and swab were detected by deflections of the recording pen. Swabs remained suspended in the male's home cage for 3 min. During the test period, an observer monitored the production of ultrasonic vocalizations produced by the male by noting the output from QMC S-100 bat detector.

*Results.* One socially experienced male exposed to intact female urine and one inexperienced male exposed to castrated male urine failed to contact the swab and did not have rhodamine in their vomeronasal organs. The dye was present in most of the remaining males who contacted the variety of types of urine (Table 1). Although fewer inexperienced males had dye in their vomeronasal organ, the effects of social experience were not significant.

In line with the results from other research (Nyby and Whitney, 1980), no ultrasonic vocalizations were detected from inexperienced males in the presence of any urine type, but some socially experienced males did emit vocalizations. The vomeronasal organ plays a crucial role as an afferent in the production of these vocalizations (Wysocki et al., 1982), but apparently, stimulation of the vomeronasal organ by urinary compounds is not a sufficient condition to elicit vocalizations: two of the mice exposed to urine from intact females, usually an adequate stimulus, had rhodamine in their vomeronasal organs, yet these males did not emit vocalizations.

The results from one mouse were intriguing. In this particular case, vocalizations were detected to female urine although rhodamine was not present in the vomeronasal organ after considerable contact (37 sec) with the swab. Either the concentration of rhodamine in the vomeronasal organ was below the

TABLE I. SUBJECTS EXHIBITING RHODAMINE-POSITIVE VOMERONASAL ORGAN AFTER CONTACT WITH URINE FROM DONOR TYPE INDICATED

| Urine donor | Inexperienced subjects | Experienced subjects |
|---|---|---|
| Male | | |
| Intact | 4/5 | 5/5 |
| Castrated | 4/4[a] | 5/5 |
| Female | | |
| Intact | 3/5 | 4/4[a] |
| Hypophysectomized | 2/5 | 4/5 |

[a] Subjects that did not contact the swab were removed.

level of detection or the mouse did not sample the nonvolatile components of the urine with his vomeronasal organ. The latter alternative is consistent with the report that urine odors elicited vocalizations from males that lacked vomeronasal organs (Wysocki et al. 1982), provided the males had had experience with females prior to surgery.

*Experiment 2*

This experiment extended the results of the previous experiment to another species and also examined the possibility that nonvolatile components of urine obtained from a species that differed from the subject also were transported to the vomeronasal organ during investigation of the heterospecific urine.

Twenty wild trapped adult male pine voles were used to assess the transport of rhodamine to the vomeronasal organ. The experiment was divided into two phases: familiarization and testing. During 8–10 days of familiarization, each vole received a cotton swab for 10 min. Ten of the voles received swabs impregnated with 1–2 drops of adult female pine vole urine, the other 10 received swabs with 1–2 drops of female meadow vole urine. This procedure served to familiarize the voles with the presence of urine-soaked swabs and ensured that all animals made contact with the swab on the day of the test.

Urine was obtained daily from individual donors by lifting each animal by the scruff of the neck. The voided urine was collected on swabs which were subsequently stored in test tubes. As needed, a swab was removed from the test tube, further wetted with 0.1 ml distilled water, and placed into the male's cage.

On the test day, food and water were removed from the cage prior to adding the swab. Each swab was then prepared with 1 drop of female urine (either meadow vole or pine vole) plus 0.1 ml of 1% rhodamine in water. Five of the males exposed to female pine vole urine during familiarization received swabs that contained female pine vole urine, the remaining five of this group received swabs that contained female meadow vole urine. Likewise, five of the males that were exposed to female meadow vole urine received swabs that contained female pine vole urine, the remaining five of this group received swabs that contained female meadow vole urine. Each swab remained with the male for 6 min.

*Results.* Rhodmaine was found in the vomeronasal organs of the majority of male pine voles (Table 2). Previous experience with female urine type (pine vole or meadow vole) or the type of rhodamine-adulterated urine used during testing (female pine vole or meadow vole) did not influence the results. We conclude that the nonvolatile dye and perhaps other nonvolatile molecules in the urine of female pine voles and meadow voles have access to receptors within the vomeronasal organs of male pine voles. Interestingly, transport of the nonvolatile marker to the vomeronasal organ occurred during presentation of both conspecific and heterospecific urine.

TABLE 2. MALES WITH RHODAMINE-POSITIVE VOMERONASAL ORGAN AFTER CONTACT WITH RHODAMINE-DYED URINE[a]

| Source of female urine experienced during familiarization | Source of female test urine | |
|---|---|---|
| | Pine voles | Meadow voles |
| Pine voles | 4/5 | 5/5 |
| Meadow voles | 4/5 | 4/5 |

[a]Males experienced the urine type indicated.

### *Experiment 3*

The above experiments demonstrated that a nonvolatile molecule placed in urine has access to the vomeronasal organ. The following experiment explored the possibility that nonvolatile molecules enter the vomeronasal organ during bouts of self-grooming. This behavior was chosen because it can be easily and reliably elicited and because it is extremely common throughout a wide range of species.

Fifteen male pine voles and 11 male meadow voles served as subjects in this experiment. Each day for at least one week prior to the test day, the fur of each vole was smeared with 0.05–0.10 cc of carboxymethylcellulose (CMC) paste, a presumably tasteless gum. The vole was then allowed to groom. On the test day, each vole had 0.05 ml of the CMC paste, containing either 0.01% ($N$ = 6 pine voles) or 0.10% rhodamine ($N$ = 9 pine voles and 11 meadow voles), applied to the fur of the flank, either on the right or left side. Each vole was then allowed to groom for 5–6 min.

*Results.* One pine vole that received 0.01% rhodamine did not groom and his vomeronasal organ did not contain rhodamine. Two of the remaining five pine voles receiving 0.01% rhodamine in the CMC paste had rhodamine in their vomeronasal organs while three did not. All nine pine voles and all but one of the meadow voles receiving 0.10% rhodamine had dye in the vomeronasal organ. Variation in the amount of dye present in the organ was evident, but was not quantified. We conclude that the nonvolatile dye and perhaps other nonvolatile molecules on the body surface have access to the vomeronasal organ during grooming.

### *Experiment 4*

As social rodents, pine and meadow voles engage in social grooming. During such encounters, nonvolatile molecules should have access to the vomeronasal organ because these types of compounds reach the vomeronasal organ during self-grooming. This hypothesis was tested in the following experiment.

Three triads of meadow voles were used. One contained three females and the others had two females and one male. On the test day, one female from each of two of the groups and the male from the third group had 0.2 ml of CMC, containing 0.10% rhodamine, applied to the fur. Each animal was then returned to its respective triad to engage in social grooming for 6 min.

*Results.* Social grooming occurred in each of the triads, but was limited to two of the three voles in each group. In the group of females, the vole that received the CMC also engaged in self-grooming and one of the females performed social grooms with her. Both of these females had rhodamine present in their vomeronasal organs.

In one of the mixed-sex groups, the male performed social grooming with the CMC-treated female. Rhodamine was present in his vomeronasal organ. The untreated female did not groom, but rhodamine was observed in her vomeronasal organ. CMC from the treated female was observed on the wall of the cage. Presumably, the rhodamine dye entered the organ of the female subsequent to her investigation of the CMC on the cage. Although the treated female engaged in bouts of self-grooming, no rhodamine was detected in her vomeronasal organ.

In the other mixed sex group, one female remained in the nest throughout the test. She did not encounter dyed CMC and her vomeronasal organ was not removed. The remaining male and female engaged in mutual social grooming. The treated male also engaged in bouts of self-grooming. Rhodamine was present in the vomeronasal organs of both animals.

The results of this experiment, together with those of experiment 3, demonstrate that nonvolatile dye, rhodamine, when placed on the fur of animals, has access to the vomeronasal organ both during self-grooming and during social grooming.

*Experiment 5*

The previous experiments demonstrated that a nonvolatile marker entered the vomeronasal organ during investigations of urine and during social and self-grooming. We next wished to determine whether access of rhodamine to the vomeronasal organ was limited to the context of social behavior or whether the nonvolatile molecule entered the vomeronasal organ during other types of behavior.

Although there are no data available implicating the vomeronasal organ in feeding by mammals, the organ appears to play a prominent role in this behavior in reptiles (Kubie and Halpern, 1976). Therefore, we determined whether a nonvolatile marker has access to the vomeronasal organ during feeding by providing voles with a familiar food that was laced with the dye on the test day.

Ten adult meadow voles (six females) and 14 adult pine voles (seven females) were used as subjects in this experiment and were provided pieces (2–3

TABLE 3. RESULTS OF FLUORESCENCE MICROSCOPY OF VOMERONASAL ORGANS OBTAINED FROM PINE AND MEADOW VOLES AFTER TREATMENT INDICATED

| Treatment | Subjects[a] | Rhodamine in vomeronasal organ |
|---|---|---|
| No rhodamine[b] | 2 mv, 1 pv | Absent in all |
| Rhodamine mouth rinse | 2 pv | Present in both |
| Rhodamine-dyed apple | 8 mv | Present in 7, absent in 1 |
| | 11 pv | Present in all |

[a] Abbreviations: mv = meadow vole; pv = pine vole.
[b] 1 mv consumed nondyed apple.

g) of apple that had been soaked overnight in 0.1% rhodamine in distilled water. Two of the meadow voles (one female) and one female pine vole were assigned to a negative control condition and did not receive rhodamine-dyed apple. One male and one female pine vole were assigned to a positive control condition and received approximately 0.03 ml of 0.1% rhodamine in deionized water as an oral lavage after consuming the apple.

*Results.* Access of the nonvolatile marker to the vomeronasal organ is not limited to the context of investigation of social cues or during social settings. As summarized in Table 3, rhodamine was observed in the vomeronasal organ after voles consumed a familiar food.

*Experiment 6*

The following experiment was conducted to further extend our observations to other species and to another consumatory behavior. In this experiment, rats (*Rattus norvegicus*) and mice were allowed to drink a dye-adulterated fluid prior to removal of their vomeronasal organs. For some of the subjects the fluid was a familiar substance, but for others the fluid was novel.

Ten adult male rats and 15 adult AKD2$F_1$/J male mice were habituated to a restricted water-access schedule to ensure drinking upon demand. Ten of the mice had daily access to 1% rhodamine in water (results from other experiments demonstrated that rhodamine was not present in the vomeronasal organ 24 hr after exposure to dye-adulterated stimuli). The remaining mice and all of the rats received only water. On the test day each animal received either 0.2% w/v saccharin (all of the rats) or water (all of the mice), each containing 1% rhodamine. Each rat was allowed 5 min to consume the dyed fluid and each mouse was allowed 10–12 min.

*Results.* Each animal consumed some of the dyed drinking solution on the test day. Rhodamine was present in the vomeronasal organ of every subject, but

there were subjective quantitative differences in its concentration. Some of the subjects consumed a familiar substance (mice drinking dyed water) and others consumed a novel substance (rats drinking saccharin). The subjects apparently sampled the fluid with their vomeronasal organs irrespective of the degree of familiarity.

*Experiment 7*

In each of the previous experiments our conclusions that nonvolatile molecules reached the vomeronasal organ were based upon the observations that a dye was found in the organ. Although nonvolatile, rhodamine is a relatively small molecule when compared with the myriad substances found in biological secretions or excretions or in nutrients. Additionally, it is possible that there is something peculiar about rhodamine which might account for its access to the vomeronasal organ. Thus, in the following experiment, we sought to determine whether a larger, very different molecule (a protein) could reach the vomeronasal organ.

A conjugate of rhodamine with bovine serum albumin (molecular weight 66,000 g/mol) was prepared by standard methods (Chen, 1969) as follows. A 100-mg sample of bovine serum albumin (Sigma, crystallized, globulin-free) was dissolved in 10 ml of 0.1 M sodium bicarbonate and cooled to 10°C. A 1.25-ml sample of 2% rhodamine isothiocyanate (Sigma, 70% labeling efficiency) in acetone was added dropwise to the stirred protein solution. The solution was stirred for 4 hr, centrifuged, placed in dialysis tubing (cutoff 12,000 g/mol), and dialyzed against 0.1 M ammonium carbonate. The retentate was chromatographed in 0.1 M ammonium carbonate buffer, pH 8.0, on a 15-mm by 60-cm Sephadex G-50 column. The conjugate eluted at the void volume and was collected. No free rhodamine was detected eluting from the column. The conjugate solution was concentrated to 3 ml by rotoevaporation at approximately 5°C and finally dialyzed against 1.6 mM sodium chloride to reduce the salt concentration. Exhaustive dialysis against water had been observed to precipitate the complex. This final solution of conjugate contained $2.65 \times 10^{-4}$ mol/liter bovine serum albumin. The dye-to-protein ratio of the conjugate was determined to be 11.7 by the method outlined by Chen (1969).

A 0.15-ml aliquot of conjugate was added to each of three 0.05-ml samples of guinea pig urine from three individual females. These dye-adulterated samples of urine were presented to three male guinea pigs. The samples were presented to the males for 60 sec with a 30-sec interval between each sample. The animals spent between 11 and 55 sec (average = 37.9 sec) with each of the samples. The animals were sacrificed, and the vomeronasal organ was removed and processed as described above.

*Results.* Although subjective quantitative differences were noted, rhoda-

mine, conjugated to albumin, was found in the vomeronasal organ of each guinea pig. We conclude that large molecules can reach the sensory epithelium of the vomeronasal organ.

*Experiment 8*

Apparently, nonvolatile molecules have access to the vomeronasal organ of a variety of species in a variety of behavioral contexts. We infer that mere nasal contact with a substrate is sufficient to initiate transport of nonvolatile molecules to the vomeronasal organ. The following experiment tested this hypothesis.

Thirteen meadow voles were used. After serving in other unrelated experiments and just after sacrifice, the rhinarium or external nares of four of the voles was painted with 0.1% rhodamine. Thereafter the presence of rhodamine in the vomeronasal organ was assessed. The bodies of the remaining nine voles were positioned in a guillotine. Two modified 30-ga. needles attached to syringes that contained 0.1% rhodamine in water were positioned at the junction between the external nares and the rhinarium. At this location, approximately 1 $\mu$l of dyed water was deposited bilaterally. The blade of the guillotine severed the neck at approximately 10, 30, or 60 sec after application of the dye. The head was then immediately frozen in liquid nitrogen, and the vomeronasal organ was removed, sectioned, and surveyed for the presence of rhodamine. The percentage of epithelium that contained dye was estimated for each vomeronasal organ.

*Results.* Unlike the dissection controls described at the beginning of the Methods and Materials section, application of rhodamine to the rhinarium or external nares resulted in transport of the dye to the vomeronasal organ in each of the animals. (Under a dissecting microscope, using other animals, we observed that liquids, placed on the lower portion of the rhinarium, moved dorsally and entered the nasal cavity. This occurred with the animal's head in its normal dorsal-ventral position. This process may assist transport of substances to the vomeronasal organ during normal sampling by the animal.)

Access of rhodamine to the vomeronasal organ was time dependent. At 10 ($N$ = 4), 30 ($N$ = 3), and 60 ($N$ = 2) sec after application of the dye, approximately 20, 60, and 100%, respectively, of the vomeronasal organ epithelium was dyed.

The vascular pump of the vomeronasal organ is nonfunctional after section of the nasopalatine nerve (Meredith et al., 1980). In its absence, fluids cannot be expelled from the vomeronasal organ. However, some hamsters that lacked both olfaction (by application of zinc sulfate to the epithelium) and the vomeronasal pump appeared able to utilize cues detected by the vomeronasal organ (Meredith et al., 1980). Among other possibilities, the authors suggested that "[t]here may be some diffusion of small amounts of stimulus substances into the VNO during the course of the...test." Our observations, i.e., nonvolatile

substances have access to the vomeronasal organ in dead animals, perhaps via capillary action, are consistent with this suggestion.

## DISCUSSION

Several recent studies (see contributions in Müller-Schwarze and Silverstein, 1980, 1983; Breipohl, 1982), as well as earlier reports (Beruter et al., 1973), have implicated large, nonvolatile molecules as vomeronasal organ stimuli. An elegant series of studies with hamsters provides a useful example. Male hamsters will mount other anesthetized males that have been anointed with hamster vaginal discharge (Singer et al., 1984). This behavior can be elicited from test males by application of a high-molecular-weight (in excess of 15,000 dalton) fraction of the vaginal discharge to the anesthetized male (Clancy et al., 1984b). However, if the test male lacks his vomeronasal organ, the fraction is ineffective (Clancy et al., 1984b).

Our prior studies demonstrated in guinea pigs that a nonvolatile molecule, the fluorescent dye rhodamine, had access to vomeronasal organ receptors when the dye was placed in the urine of a female and the conspecific male was allowed to contact the urine–dye mixture (Wysocki et al., 1980a). The studies reported herein have extended these earlier findings by (1) demonstrating a similar phenomenon in other species; (2) demonstrating that the nonvolatile marker has access during grooming, feeding, and drinking; and (3) demonstrating that a large, proteinaceous molecule also reaches the epithelium of the vomeronasal organ. Apparently, contact with substances dyed with rhodamine or a rhodamine–BSA conjugate results in transfer of nonvolatile molecules to the vomeronasal ogan.

The ease with which various nonvolatile molecules reach the vomeronasal organ may vary substantially. Rhodamine is small and lipophilic, characteristics that may facilitate movement through mucus. However, BSA–rhodamine conjugate, a much larger molecule with different physical chemical properties, also reached the vomeronasal organ epithelium, suggesting that our findings cannot be due exclusively to special characteristics of rhodamine. Furthermore, transport of other nonvolatile markers has been demonstrated in other laboratories (Ladewig and Hart, 1980; Schilling, 1980).

The results from this series of experiments lead us to conclude that, like olfactory receptors, the vomeronasal epithelium appears to maintain almost constant contact with the environment. Presumably, the vascular pump described by Meredith and O'Connell (1979) aides in this regard.

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# FLAVONOID PIGMENTS IN MARBLED WHITE BUTTERFLY (*Melanargia galathea*) ARE DEPENDENT ON FLAVONOID CONTENT OF LARVAL DIET

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**Abstract**—Analyses of two-dimensional chromatographic flavonoid patterns of butterflies reared on different grass species have shown that the flavonoid pattern of *Melanargia galathea* is dependent on the flavonoid content of the larval diet. This confirms the dietary origin of flavonoid pigments in *M. galathea*. The flavonoid patterns of butterflies reared on different grass species differ from each other and from the larval food plants; *M. galathea* reared on the same grass species have identical flavonoid patterns. Differences in the butterfly and larval food plant flavonoid patterns indicate that the ingested flavonoids are metabolized by *M. galathea* or its gut flora before sequestration. The distinct flavonoid patterns of butterflies reared on different larval food plants have been defined as the flavonoid fingerprint profiles for each grass species. Similarity between the *Festuca rubra* flavonoid fingerprint profile and the constant flavonoid pattern characteristic of wild-captured *Melanargia* suggests that *Melanargia* larvae are not generalist grass feeders, but are specific to *F. rubra* or to *F. rubra* and a few closely related grass species in the wild.

**Key Words**—*Melanargia galathea*, Lepidoptera, Satyridae, marbled white butterfly, Gramineae, two-dimensional paper chromatography, flavonoids, flavonoid fingerprints, larval food plant specificity, insect-plant interactions, chemical ecology.

## INTRODUCTION

The occurrence of flavonoids, a class of phenolic plant pigments, in the plant kingdom has been well documented (Harborne and Mabry, 1982). Although present in other plant groups, they are most abundant in the angiosperms, often accumulating in the living tissues to the extent of between 5 and 30% of the dry



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weight. Flavonoids have also been recognized in insects on a number of occasions (Thomson, 1926; Ford, 1941, 1944; Fujimoto et al., 1959; Hayashiya et al., 1959; Morris and Thomson, 1963, 1964; Feltwell and Valadon, 1970), but in the main, identification of insect flavonoids has either not been attempted or is subject to doubt.

Morris and Thomson (1964) provided the first positive identification of flavonoids in the animal kingdom. From the wing extracts of 400 marbled white butterflies (*M. galathea* L.), they identified tricin, tricin glycosides, and an orientin glycoside. More recently, 18 flavonoids have been identified in *M. galathea* (Wilson, 1985). Furthermore, analyses of paper chromatographic flavonoid patterns of individual *M. galathea*, *M. galathea* var *procida*, M. *larissa*, *M. ines*, *M. lachesis*, *M. russiae*, and *M. occitanica* butterflies have shown that they contain the same flavonoids and that a constant flavonoid pattern exists for these species (Wilson, 1985). These flavonoids have also been identified in the egg, larval, and pupal stages of *M. galathea*, with the exception of the incompletely identified tricin 4′-conjugate which was absent from the eggs and the first-instar larvae before feeding on the larval food plants begins (Wilson, 1985).

Flavonoid pigments in insects are believed to be of a dietary origin, obtained in butterflies by the larval feeding habits. As the larvae of *Melanargia* are thought to feed on a variety of grass species, each containing different flavonoids, the constant flavonoid pattern may be produced by either selectively sequestering flavonoids from the diet and/or metabolizing dietary flavonoids to form those characteristic of the butterfly. Alternatively, the larvae may not be generalist grass feeders but specific to one or a small number of closely related grass species.

To determine the way(s) in which the constant *Melanargia* flavonoid pattern may be produced in the wild, the relationship between flavonoid pigments in *M. galathea* and its larval food plants has been investigated. In this paper the flavonoid patterns of butterflies reared on different grass species have been compared with each other and with those of the larval food plants by two-dimensional paper chromatography and analyses of the flavonoid aglycones.

## METHODS AND MATERIALS

*Melanargia galathea, Larvae.* Live *M. galathea* were collected from a site at North Moreton in Oxfordshire, during July and August 1981, and transported to the laboratory in cardboard containers. Female butterflies laid eggs freely when fed on diluted honey and maintained in 1 × 1-m cages, constructed of wooden frames and white netting, at room temperature in the laboratory. The eggs were collected daily and placed in Petri dishes with buff colored pieces of grass, onto which the larvae crawl shortly after hatching and begin diapausing.

*Larval Food Plants.* Twenty-five grass species (Table 2) were selected from

those commonly found in the *Melanargia* habitat, for use as experimental larval food plants. The grasses were grown from seed sown in sterilized soil in 15-cm-diameter plant pots to produce a sward of each species. Details of the seed sources are available from the author on request. Identities of the grass species were verified by standard techniques (Hubbard, 1968) by the author and voucher specimens were deposited in the University of Reading Herbarium.

*Larval Rearing Containers.* Cylinders of clear plastic, 60 cm in height, were constructed and placed over the tops of the plant pots containing the grasses. The lower end of each cylinder was firmly attached to the rim of each pot using autoclaving tape chosen for its water resistance.

*Methods.* Fifty-five diapausing first-instar larvae were placed in each food plant container. Larvae were transferred from the Petri dishes to the containers while still attached to the buff-colored pieces of grass to minimize damage to them caused by handling. The tops of the containers were sealed with white netting fastened by a rubber band, and the containers placed in a cold frame in the University of Reading Botanic garden during September. Larvae were reared outside under as near natural conditions as possible to reduce the effects that temperature or day length might have on the flavonoid contents of the butterflies.

During May when the larvae were full grown and ready to pupate, the containers were moved to a glasshouse so that the pupation and emergence of butterflies could be monitored. Shortly after emergence, butterflies were removed from the container, killed using ethyl acetate vapor, and their flavonoids examined.

The wings and body of each butterfly were separated, placed in small sample tubes, and the flavonoids extracted by soaking the crushed tissues in 1 ml of 70% ethanol for 12 hr at room temperature.

Extracts of the grass species were produced by immersing approximately 20 g of fresh leaf material, cut into small pieces, in about 50 ml of boiling 70% ethanol, and boiling for a further 3 min. After cooling, the ethanolic plant material was soaked for 12 hr in a stoppered flask at room temperature.

Concentrated aliquots of the plant and insect extracts were applied to $\frac{1}{4}$ sheets of Whatman No. 1 chromatography paper, and run two-dimensionally in BAW (*n*-butanol–acetic acid–water, 4:1:5, upper phase) and 15% aqueous HOAc (acetic acid) (Harborne, 1973). Dried chromatograms were examined in long-wave UV light in the absence and presence of ammonia vapor, and the position and color of each spot were recorded.

The position of flavonoid spots on the 2-D chromatograms is a good indication of the nature of the flavonoids present (Harborne, 1973). Flavonoids occupying different positions on the chromatograms are different; however, flavonoids occupying the same positions are not necessarily the same since some compounds have similar mobilities in BAW and 15% HOAc. The identification and cochromatography of flavonoids occupying similar positions on the 2-D chromatograms is required to confirm that they are the same compound.

Flavone aglycones, most flavone glycosides, and flavonol 3-glycosides appear as dark spots on the papers. After fuming with ammonia, most of these spots turn yellow or yellow green, but some flavonoids including 6-hydroxy flavones and flavone 4′-glycosides remain dark. Flavonol aglycones and flavonol 7-glycosides are yellow and remain so when fumed with ammonia. Flavone 5-glycosides are light blue or white and turn fluorescent yellow or yellow green in the presence of ammonia.

A portion of each plant and insect extract was hydrolyzed with 2 N HCl at 100°C for 30–40 min, the cooled hydrolyzate extracted twice with ethyl acetate, the extracts combined, evaporated to dryness, and the residues dissolved in a few drops of 90% ethanol. Concentrated spots of the residues were applied to Whatman No. 1 chromatography paper and the aglycones identified by their $R_f$ values in five solvents: BAW (4:1:5); PhOH (phenol–water, 4:1); CAW (chloroform–acetic acid–water, 30:15:2); FOR (acetic acid–conc.HCl–water, 30:3:1); and 15% HOAc; their colors in UV light; and by comparison with authentic samples. Flavone *C*-glycosides were detected by 4-hr hydrolysis with 2 N HCl at 100°C, extraction in isoamyl alcohol, and paper chromatography against authentic markers in BAW, $H_2O$, PhOH, and 15% HOAc (Harborne, 1967, 1973).

The tricin 4′-conjugate was detected by paper electrophoresis on Whatman No. 3 paper, at pH 2.2 (7.5% acetic acid, 2.5% formic acid buffer, 1:1) for 2 hr, at 400 V/cm with an authentic marker, quercetin 3-sulfate (Harborne et al., 1975). In UV light the conjugate appears on the dried papers as a dark absorbing spot, with an electrophoretic mobility of 1.0 compared to that of quercetin 3-sulfate.

*Examination of M. galathea Feces for Flavonoids.* Three third-instar larvae feeding on *Molinea caerulea* L. Moench were removed from their container in April, placed in a small circular Perspex dish, and maintained in the laboratory at room temperature. Fresh *M. caerulea* was supplied to the larvae daily, excess plant material from the previous day removed, and the feces collected and placed in small sample tubes. After one week, the larvae were returned to their container.

The feces were extracted by soaking in 2 ml of 70% ethanol for 24 hr at room temperature, and the flavonoid content examined by two-dimensional chromatography in BAW and 15% HOAc.

## RESULTS

Survival of *M. galathea* on the experimental larval food plant species was very low; adult butterflies emerged on only seven of the 25 grass species, namely, *Lolium perenne* L., *Phleum pratense* L., *Agrostis tenuis* Sibth., *Festuca praten-*

TABLE 1. NUMBERS OF IMAGOS PRODUCED AND SURVIVAL OF *M. galathea*, EXPRESSED AS PERCENTAGE OF 55 FIRST-INSTAR LARVAE PLACED ON EACH FOOD PLANT AT BEGINNING OF EXPERIMENT, FOR SEVEN GRASS SPECIES ON WHICH *M. galathea* BUTTERFLIES EMERGED

| Larval food plant species | Numbers of *M. galathea* imagos produced | Percent of *M. galathea* surviving |
|---|---|---|
| *Festuca arundinacea* | 3 | 5.5 |
| *Festuca pratensis* | 2 | 3.6 |
| *Lolium perenne* | 5 | 9.1 |
| *Phleum pratense* | 3 | 5.5 |
| *Agrostis tenuis* | 5 | 9.1 |
| *Bromus erectus* | 3 | 5.5 |
| *Festuca rubra* | 15 | 27.3 |

*sis* Huds., *F. arundinacea* Schreb., *F. rubra* L., and *Bromus erectus* Huds. When expressed as a percentage of the 55 first-instar larvae placed on each food plant at the beginning of the experiment (Table 1), the survival of *M. galathea* on six of these species was less than 10%. *M. galathea* survived the best on *F. rubra* with 27.3% of the larvae producing imagos.

First-instar larvae failed to feed on *Holcus lanatus* L., *H. mollis* L., *Dactylis glomerata* L., *Nardus stricta* L., *Arrhenatherum elatius* (L.) Beauv. ex J. & C. Presl., and *Cynosurus cristatus* L., but remained attached to the edges of the blades of grass and subsequently died in this position. Attempts to rear *M. galathea* on these grass species the following year gave the same results.

On the remaining 12 species of grass, larvae were observed to feed, but full-grown larvae and pupae were produced only on *Agrostis stolonifera* L., *Molinea caerulea*, *Alopecurus pratensis* L., and *Poa pratensis* L., although adults failed to emerge from the pupae. A summary of the development of *M. galathea* on each grass species is presented in Table 2.

Examples of the paper chromatograms run in BAW and 15% HOAc of the seven grass species and of *M. galathea* reared on them are reproduced in Figure 1. $R_f$ data and color characteristics of flavonoids in the spots on these chromatograms are given in Table 3.

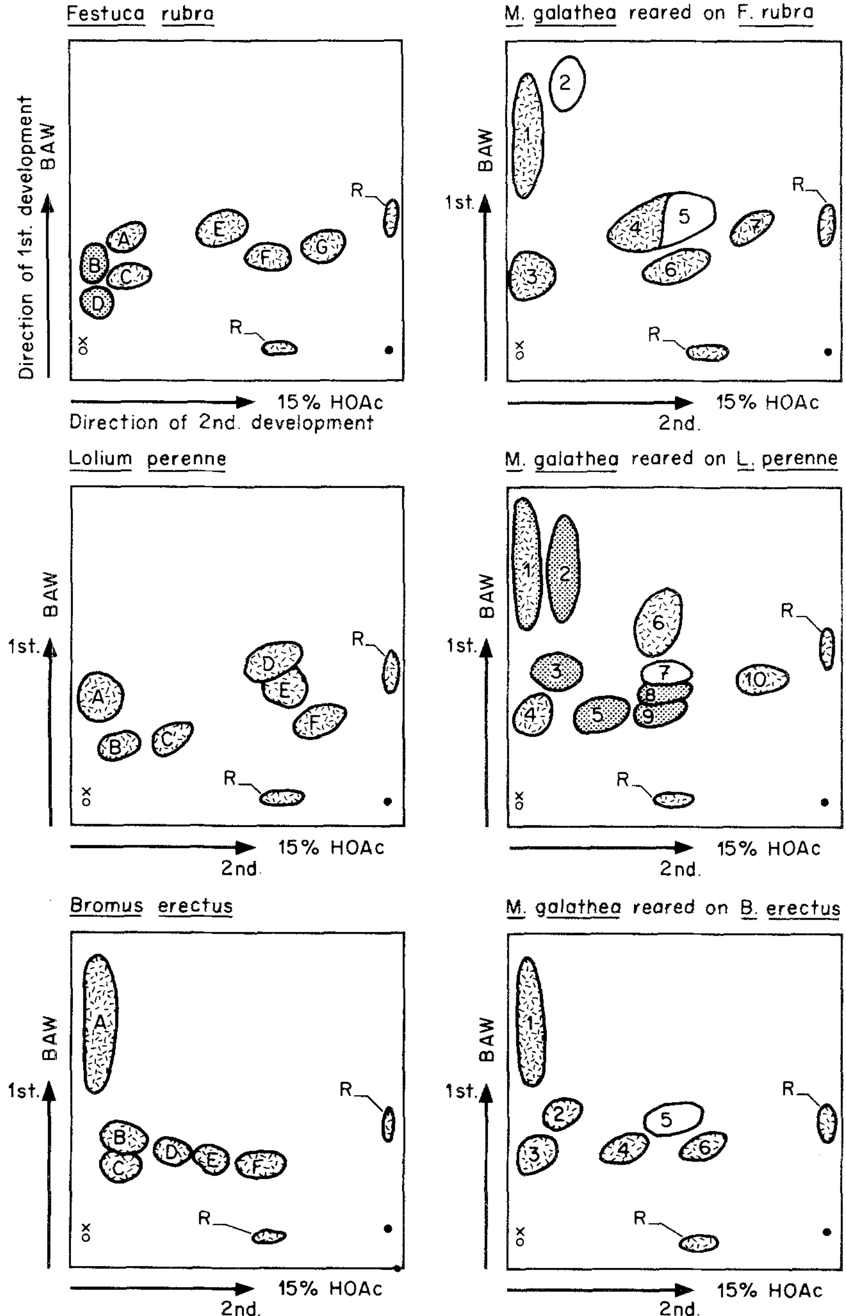
The flavonoid patterns of butterflies reared on different grass species differed from each other and from the larval food plant species. *M. galathea* reared on the same grass species have identical flavonoid patterns. Because flavonoid spots occupying different positions on the chromatograms contain different compounds, differences in the plant and insect flavonoid patterns indicate that they

TABLE 2. DEVELOPMENT OF *M. galathea* ON 25 SPECIES OF GRASS SELECTED AS EXPERIMENTAL LARVAL FOOD PLANTS

| Grass species | *M. galathea* development |
|---|---|
| *Arrhenatherum elatius* (L.) Beauv. ex J. & C. Presl.; *Cynosurus cristatus* L.; *Dactylis glomerata* L., *Holcus lanatus* L.; *Holcus mollis* L.; *Nardus stricta* L. | First-instar larvae did not feed, but remained attached to the edges of blades of grass and died in this position. |
| *Agropyron repens* (L.) Beauv., *Anthoxanthum odoratum* L.; *Briza maxima* L., *Bromus mollis* L.; *Bromus sterilis* L.; *Deschampsia flexuosa* (L.) Trin.; *Festuca ovina* L.; Poa annua L. | Larvae fed on these grasses but did not produce full-grown larvae or pupae. |
| *Agrostis stolonifera* L.; *Alopercurus pratensis* L.; *Molinea caerulea* (L.) Moench; *Poa pratensis* L. | Pupae were produced but adults did not emerge. |
| *Agrostis tenuis* Sibth.; *Bromus erectus* Huds.; *Festuca arundinacea* Schreb.; *Festuca pratensis* Huds.; *Festuca rubra* L.; *Lolium perenne* L.; *Phleum pratense* L. | Imagos emerged from pupae. |

contain different flavonoids. Similarly the identical flavonoid patterns of butterflies reared on the same grass species indicate that they contain the same flavonoids. From the two-dimensional chromatographic flavonoid patterns, it is possible to say that flavonoids occupying different positions on the plant and insect chromatograms are definitely different, but not to say categorically that flavonoids with the same color characteristics and occupying similar positions on the chromatograms are the same. For this reason the overall differences/similarities in the two-dimensional chromatographic flavonoid patterns of *M. galathea* and its larval food plants should be viewed rather than individual flavonoid spots on the chromatograms. Identification of the plant and insect flavonoids, although not possible in this experiment as insufficient quantities of

FIG. 1. Two-dimensional paper chromatographic patterns of the grass larval food plant species and of *M. galathea* reared on them. Chromatograms were developed first in BAW (4:1:5), then in 15% aqueous HOAc. Spot color characteristics in longwave ultraviolet light are represented as follows: dark absorbing spots changing to yellow in the presence of ammonia vapor, lightly stippled; blue spots changing to yellow in the presence of ammonia, hatched; dark absorbing spots remaining so when fumed with ammonia, unstippled; yellow spots remaining so when fumed with ammonia, heavily stippled. ×, represents the orgin; •, the position at which the rutin marker was applied for development of the chromatogram in BAW; ○, the position at which the rutin marker was applied for development of the chromatogram in 15% HOAc.


Festuca rubra
M. galathea reared on F. rubra
Lolium perenne
M. galathea reared on L. perenne
Bromus erectus
M. galathea reared on B. erectus
Direction of 1st. development BAW
1st. BAW
15% HOAc
Direction of 2nd. development
2nd.
A
B
C
D
E
F
G
R
1
2
3
4
5
6
7
8
9
10

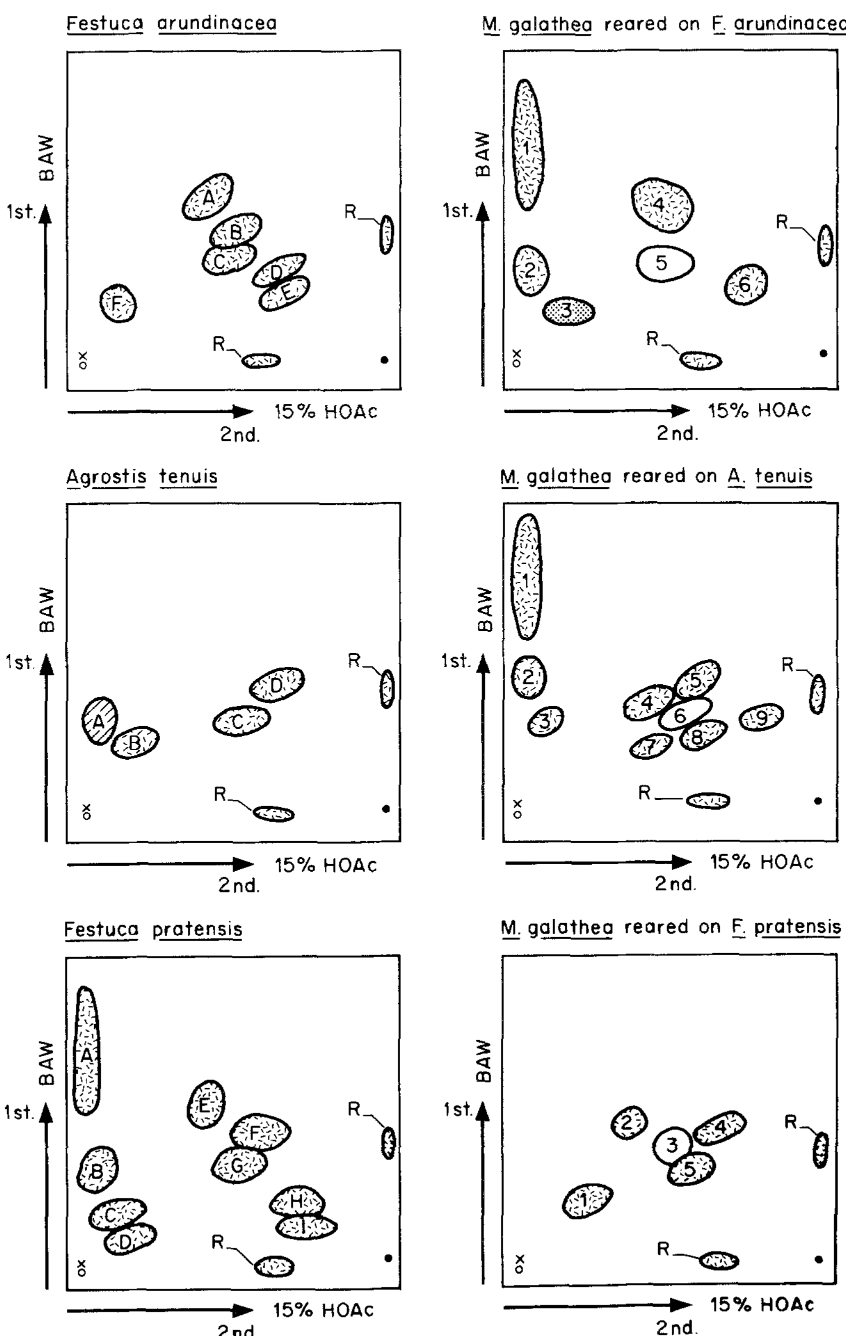



FIG. 1. Continued

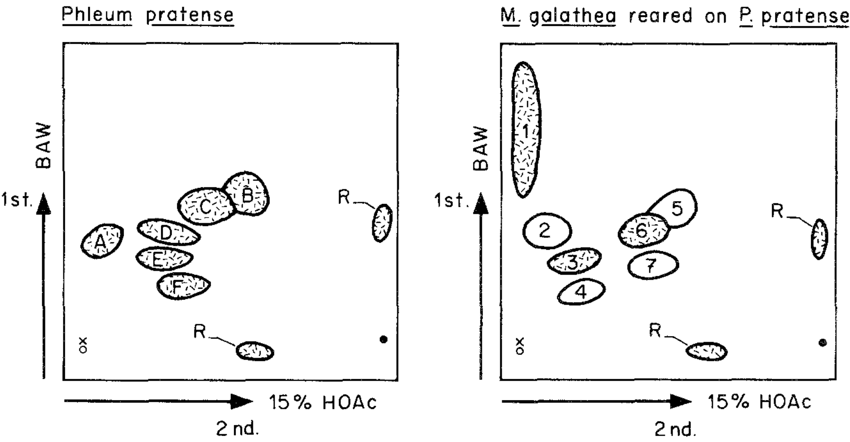



FIG. 1. Continued

adult butterflies emerged on each grass species for analysis of their flavonoids, would provide support for these qualitative results.

Of the different flavonoid patterns, only that of *M. galathea* reared of *F. rubra* was similar to the constant flavonoid pattern of wild-captured *M. galathea* (see Figures 1 and 2, and Tables 3 and 4). The flavonoid pattern of *M. galathea* reared on *F. ovina* L. was not determined, as severe aphid attacks in two successive years destroyed the plants and the larvae feeding on them.

The flavonoid aglycones, produced in the butterflies by acid hydrolysis, were the same as those in their larval food plant species (Table 5). In addition to the flavones and glycoflavones characteristic of wild *M. galathea*, the flavonol quercetin was identified in *F. arundinacea*, and both quercetin and kaempferol in *L. perenne* and *F. pratensis*.

Prior to acid hydrolysis, free tricin was tentatively identified in all the experimental butterflies but in only two of the larval food plant species, namely, *Bromus erectus* and *F. pratensis* (Table 3).

Tricin 4′-conjugate was absent from all the larval food plant species but present in all the butterflies reared on them (Table 5). Tricin 4′-glucoside was tentatively identified in only those butterflies reared on *F. rubra* (Table 3).

Although the flavonoid pattern of the feces of *M. galathea* larvae feeding on *M. caerulea* is more simple than that of the plant, it is composed solely of compounds found in the plant (see Figure 3 and Table 6). From the position and color characteristics of flavonoids on the two-dimensional chromatograms, spot D on the plant chromatogram appears to be the same as spot 2 on that of the insect feces; spot E the same as spot 3; spot A the same as spot 1; and spot J the same as spot 4. On the basis of its position and color characteristics, the flavonoid in spots D and 2 on the plant and insect chromatograms, respectively, has been tentatively identified as tricin 5-glucoside.

TABLE 3. $R_f$ DATA AND COLOR CHARACTERISTICS FOR FLAVONOID SPOTS ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS OF LARVAL FOOD PLANT SPECIES AND *M. galathea* REARED ON THEM[a]

| | $R_f$ (×100) in | | | |
|---|---|---|---|---|
| Spot | BAW | 15% HOAc | COL | Tentative identification |
| *Festuca rubra* | | | | |
| A | 38 | 16 | D/Y | |
| B | 30 | 09 | D/Y | |
| C | 25 | 23 | D/Y | |
| D | 16 | 08 | B/Y | |
| E | 44 | 44 | D/Y | |
| F | 30 | 58 | D/Y | |
| G | 36 | 75 | D/Y | |
| R | 45 | 62 | D/Y | |
| *M. galathea* reared on *F. rubra* | | | | |
| 1 | 70 | 04 | D/Y | Tr, Lut |
| 2 | 84 | 19 | D/D | Tr 4′-gluc |
| 3 | 33 | 08 | D/Y | Tr 7-gluc |
| 4 | 47 | 38 | D/Y | Or 7-gluc, Isoor, Vit 7-gluc, Lut 7-digluc |
| 5 | 47 | 55 | D/D | Tricin 4′-conjugate |
| 6 | 33 | 48 | D/Y | Lut 7-trigluc, Isoor 7-gluc, |
| 7 | 42 | 60 | D/Y | Isovit 7-gluc |
| R | 44 | 60 | D/Y | |
| *Lolium perenne* | | | | |
| A | 35 | 11 | D/Y | |
| B | 19 | 18 | D/Y | |
| C | 19 | 30 | D/Y | |
| D | 50 | 58 | D/Y | |
| E | 41 | 63 | D/Y | |
| F | 25 | 71 | D/Y | |
| R | 42 | 60 | D/Y | |
| *M. galathea* reared on *L. perenne* | | | | |
| 1 | 78 | 04 | D/Y | Tricin |
| | | | Y/Y | Kaempferol |
| 2 | 73 | 14 | Y/Y | — |
| 3 | 45 | 16 | Y/Y | — |
| 4 | 34 | 08 | D/Y | — |
| 5 | 33 | 24 | Y/Y | — |
| 6 | 62 | 46 | D/Y | — |

TABLE 3. Continued

| Spot | $R_f$ (×100) in BAW | 15% HOAc | COL | Tentative identification |
|---|---|---|---|---|
| *M. galathea* reared on *L. perenne* | | | | |
| 7 | 43 | 46 | D/D | Tricin 4′-conjugate |
| 8 | 39 | 47 | Y/Y | — |
| 9 | 32 | 43 | Y/Y | — |
| 10 | 41 | 73 | D/Y | — |
| R | 41 | 58 | D/Y | |
| *Bromus erectus* | | | | |
| A | 76 | 03 | D/Y | |
| B | 38 | 14 | D/Y | |
| C | 26 | 11 | D/Y | |
| D | 33 | 27 | D/Y | |
| E | 31 | 38 | D/Y | |
| F | 31 | 57 | D/Y | |
| R | 41 | 65 | D/Y | |
| *M. galathea* reared on *B. erectus* | | | | |
| 1 | 77 | 05 | D/Y | Tricin |
| 2 | 48 | 23 | D/Y | — |
| 3 | 36 | 11 | D/Y | — |
| 4 | 36 | 32 | D/Y | — |
| 5 | 42 | 49 | D/D | Tricin 4′-conjugate |
| 6 | 32 | 54 | D/Y | — |
| R | 41 | 54 | D/Y | |
| *Festuca arundinacea* | | | | |
| A | 58 | 37 | D/Y | |
| B | 45 | 51 | D/Y | |
| C | 38 | 47 | D/Y | |
| D | 31 | 61 | D/Y | |
| E | 25 | 64 | D/Y | |
| F | 20 | 14 | D/Y | |
| R | 39 | 56 | D/Y | |
| *M. galathea* reared on *F. arundinacea* | | | | |
| 1 | 71 | 03 | D/Y | — |
| 2 | 34 | 08 | D/Y | — |
| 3 | 22 | 24 | Y/Y | — |
| 4 | 56 | 44 | D/Y | — |
| 5 | 35 | 45 | D/D | Tricin 4′-conjugate |
| 6 | 28 | 73 | D/Y | — |
| R | 35 | 56 | D/Y | |

TABLE 3. Continued

| Spot | $R_f$ (×100) in BAW | $R_f$ (×100) in 15% HOAc | COL | Tentative identification |
|---|---|---|---|---|
| *Agrostis tenuis* | | | | |
| A | 29 | 08 | B/Y | |
| B | 22 | 21 | D/Y | |
| C | 29 | 50 | D/Y | |
| D | 40 | 62 | D/Y | |
| R | 43 | 59 | D/Y | |
| *M. galathea* reared on *A. tenuis* | | | | |
| 1 | 79 | 03 | D/Y | Tricin |
| 2 | 45 | 04 | D/Y | — |
| 3 | 29 | 11 | D/Y | — |
| 4 | 38 | 38 | D/Y | — |
| 5 | 42 | 56 | D/Y | — |
| 6 | 32 | 51 | D/D | Tricin 4′-conjugate |
| 7 | 24 | 43 | D/Y | — |
| 8 | 26 | 59 | D/Y | — |
| 9 | 30 | 77 | D/Y | — |
| R | 35 | 61 | D/Y | |
| *Festuca pratensis* | | | | |
| A | 74 | 04 | D/Y | |
| B | 36 | 07 | D/Y | |
| C | 21 | 15 | D/Y | |
| D | 11 | 20 | D/Y | |
| E | 62 | 41 | D/Y | |
| F | 48 | 56 | D/Y | |
| G | 33 | 50 | D/Y | |
| H | 23 | 74 | D/Y | |
| I | 15 | 76 | D/Y | |
| R | 43 | 56 | D/Y | |
| *M. galathea* reared on *F. pratensis* | | | | |
| 1 | 25 | 22 | D/Y | — |
| 2 | 50 | 39 | D/Y | — |
| 3 | 42 | 46 | D/D | Tricin 4′-conjugate |
| 4 | 48 | 64 | D/Y | — |
| 5 | 31 | 54 | D/Y | — |
| R | 39 | 66 | D/Y | |
| *Phleum pratense* | | | | |
| A | 38 | 08 | D/Y | |
| B | 57 | 52 | D/Y | |
| C | 50 | 41 | D/Y | |

TABLE 3. Continued

| Spot | $R_f$ (×100) in BAW | $R_f$ (×100) in 15% HOAc | COL | Tentative identification |
|---|---|---|---|---|
| *Phleum pratense* | | | | |
| D | 43 | 33 | D/Y | |
| E | 36 | 31 | D/Y | |
| F | 26 | 37 | D/Y | |
| R | 41 | 58 | D/Y | |
| *M. galathea* reared on *P. pratense* | | | | |
| 1 | 82 | 05 | D/Y | Tricin |
| 2 | 48 | 19 | D/D | — |
| 3 | 35 | 28 | D/Y | — |
| 4 | 27 | 27 | D/D | — |
| 5 | 51 | 49 | D/D | — |
| 6 | 45 | 42 | D/Y | — |
| 7 | 35 | 44 | D/D | Tricin 4′-conjugate |
| R | 37 | 60 | D/Y | |

[a]Abbreviations: COL = color in longwave ultraviolet light in the absence and pressence of ammonia vapor; D = dark absorbing; B = blue; Y = yellow; bAW = butanol-acetic acid-water, 4:1:5, upper phase; 15% HOAc = 15% aqueous acetic acid; R = rutin marker.

## DISCUSSION

The results of this investigation have clearly demonstrated that the flavonoid pattern of *M. galathea* is dependent on the flavonoid content of its larval food plants and that flavonoid pigments in this insect are of a dietary origin.

From differences in the flavonoid patterns of *M. galathea* and its larval food plants, it is evident that the ingested flavonoids are not sequestered unaltered, but are first metabolized by the butterfly or its gut flora. However, a comparison of the flavonoid pattern of *Molinea caerulea* with that of the feces of *M. galathea* larvae feeding on *M. caerulea* reveals that not all of the ingested flavonoids are metabolized and sequestered; some are excreted unaltered. The presence of tricin in the feces suggests that a number of pathways exist for this compound when ingested by *M. galathea*; excretion, sequestration, or metabolism and then sequestration. Flavonoid metabolism and excretion as nonflavonoid fragments by *M. galathea* has yet to be determined.

Although the metabolism of flavonoids before sequestration is reported here for the first time, the metabolism of other classes of secondary plant compound

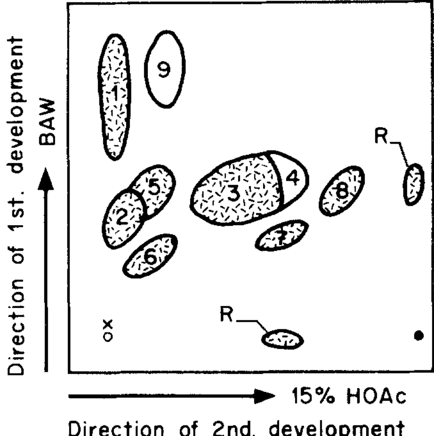



FIG. 2. The two-dimensional paper chromatographic pattern of wild-captured *M. galathea*. Chromatographic conditions and spot color characteristics are as given for Figure 1.

before sequestration by insects is frequently recognized. Carotenoids absent from the larval food plants have been identified in a number of moths and papilionid butterflies (Harashima et al., 1972, 1976; Kayser, 1982); pyrrolizidine alkaloid metabolites have been identified in *Arctia caja* L. and *Tyria jacobeaea* L. feeding on *Senecio vulgaris* L. and *S. jacobaea* L. (Aplin and Rothschild, 1972), and in the hairpencils of *Danaus plexippus* L. (Edgar and Culvenor, 1974; Edgar et al., 1974); while some cardenolides from *Asclepias eriocarpa* Benth., and *A. curassavica* L. are altered by *Danaus plexippus* before storage (Roeske et al., 1976; Brower et al., 1982).

The different flavonoid patterns of butterflies reared on different grass species, but their identical patterns when reared on the same species, suggest that the constant flavonoid pattern of wild-captured *M. galathea* must be produced by the larvae feeding on the same grass species. Support for a less general larval diet than previously thought is provided by the absence of flavonols (3-hydroxy flavones) from wild-captured butterflies, despite an ability to sequester them, demonstrated by the presence of quercetin and kaempferol in some of the experimental butterflies, thereby eliminating a number of flavonol-containing grasses in the *Melanargia* habitat from the diet.

Similarities between the flavonoid pattern of wild-captured *M. galathea* and that of *M. galathea* reared on *F. rubra* suggest that the flavonoid content of *F. rubra* is similar to that of grasses selected as larval food plants in the wild. Particularly interesting is the appearance of tricin 4′-glucoside, a flavonoid char-

TABLE 4. $R_f$ DATA AND IDENTITIES OF FLAVONOIDS ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS OF WILD-CAPTURED *M. galathea*[a]

| | $R_f$ (×100) in | | | |
|---|---|---|---|---|
| Spot | BAW | 15% HOAc | Color in UV (−/+ $NH_3$) | Flavonoid components |
| 1 | 71 | 07 | D/Y | Tricin, luteolin, apigenin |
| 2 | 35 | 10 | D/Y | Tricin 7-glucoside, orientin |
| 3 | 46 | 41 | D/Y | Luteolin 7-diglucoside, orientin 7-glucoside, vitexin 7-glucoside, isoorientin, isovitexin |
| 4 | 41 | 54 | D/D | Tricin 4′-conjugate |
| 5 | 49 | 16 | D/Y | Luteolin 7-glucoside, apigenin 7-glucoside |
| 6 | 23 | 20 | D/Y | Tricin 7-diglucoside |
| 7 | 31 | 45 | D/Y | Luteolin 7-triglucoside, isoorientin 7-glucoside |
| 8 | 37 | 68 | D/Y | Isovitexin 7-glucoside |
| 9 | 80 | 22 | D/Y | Tricin 4′-glucoside |
| R | 41 | 57 | D/Y | Rutin marker |

[a] Abbreviations: D/Y, dark absorbing spot on the chromatogram in longwave ultraviolet light, changing to yellow in the presence of ammonia vapor; D/D, dark absorbing spot remaining so in the presence of ammonia vapor. BAW, *n*-butanol–acetic acid–water, 4:1:5, upper phase; 15% HOAc, 15% aqueous acetic acid.

acteristic of wild *Melanargia*, in only those butterflies reared on *F. rubra*. Together, the similarity in flavonoid patterns and the favorable survival of *Melanargia* on this species suggest that *M. galathea* is probably specific to *F. rubra* or to *F. rubra* and a small number of closely related grass species in the wild.

Although rearing *M. galathea* on *F. ovina* was unsuccessful in this experiment, this butterfly has been reported to feed on *F. ovina* on a number of occasions, even when this grass is relatively rare at a particular site. It is possible therefore that *F. ovina* is one of the preferred larval food plants of *M. galathea*.

The relationship between flavonoids in *M. galathea* and its graminous larval food plants bears a distinct resemblance to that between cardenolides in *D. plexippus* and its asclepiad larval food plants. Just as different grass species produce different paper chromatographic flavonoid patterns in *M. galathea*, so different *Asclepias* species are responsible for distinctive thin-layer chromato-

TABLE 5. OCCURRENCE OF TRICIN 4′-CONJUGATE AND FLAVONOID AGLYCONES IN GRASS SPECIES AND IN *M. galathea* REARED ON THEM[a]

| Larval food plant species | Plant | | *M. galathea* reared on grass species | |
|---|---|---|---|---|
| | Tricin 4′-conjugate | Aglycones[b] | Tricin 4′-conjugate | Aglycones[b] |
| *Festuca arundinacea* | − | Tr, Qu | + | Tr, Qu |
| *Festuca pratensis* | − | Tr, Qu, Km | + | Tr, Qu, Km |
| *Lolium perenne* | − | Tr, Qu, Km | + | Tr, Qu, Km |
| *Phleum pratense* | − | Tr, Or | + | Tr, Or |
| *Agrostis tenuis* | − | Tr, Or | + | Tr, Or |
| *Bromus erectus* | − | Tr, Lut, Isovit | + | Tr, Lut, Isovit |
| *Festuca rubra* | − | Tr, Lut, Or, Isoor, Vit, Isovit | + | Tr, Lut, Or, Isoor, Vit, Isovit |

[a] Abbreviations: Tr, tricin; Lut, luteolin; Qu, quercetin; Km, kaempferol; Or, orientin; Vit, vitexin; Isovit, isovitexin; Isoor, isorientin. +, compound present; −, compound absent.
[b] Flavonoid aglycones were produced by hydrolysis of the plant and insect extracts with 2 N HCl at 100°C for 30 min.

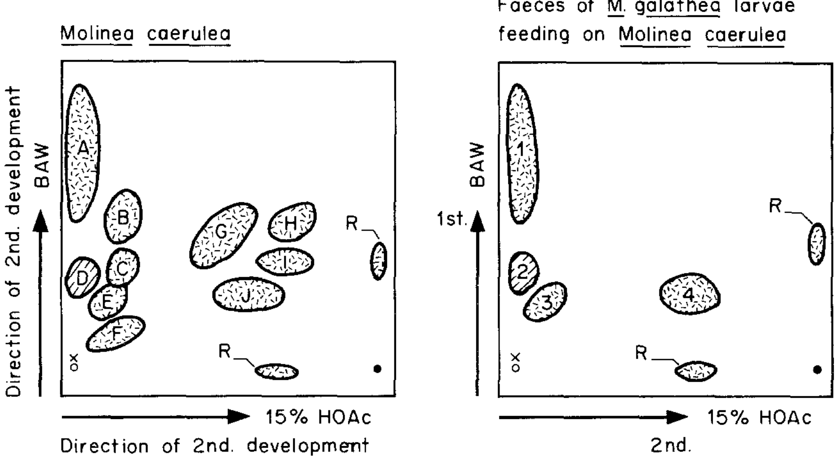



FIG. 3. Two-dimensional paper chromatographic patterns of *Molinea caerulea* and of the feces of *M. galathea* larvae feeding on *M. caerulea*. Chromatographic conditions and spot color characteristics are as given for Figure 1.

TABLE 6. $R_f$ DATA AND COLOR CHARACTERISTICS FOR FLAVONOID SPOTS ON TWO-DIMENSIONAL PAPER CHROMATOGRAMS OF *Molinea caerulea* AND FECES OF *M. galathea* FEEDING ON *M. caerulea*[a]

| | $R_f$ (×100) in | | | |
|---|---|---|---|---|
| Spot | BAW | 15% HOAc | COL | Tentative identification |
| *Molinea caerulea* | | | | |
| A | 70 | 03 | D/Y | |
| B | 54 | 24 | D/Y | |
| C | 34 | 22 | D/Y | |
| D | 31 | 08 | B/Y | |
| E | 23 | 16 | D/Y | |
| F | 17 | 22 | D/Y | |
| G | 47 | 46 | D/Y | |
| H | 54 | 75 | D/Y | |
| I | 38 | 68 | D/Y | |
| J | 30 | 54 | D/Y | |
| R | 42 | 64 | D/Y | |
| Feces of *M. galathea* larvae feeding on *M. caerulea* | | | | |
| 1 | 74 | 03 | D/Y | Tricin |
| 2 | 33 | 08 | B/Y | Tricin 5-glucoside |
| 3 | 23 | 14 | D/Y | — |
| 4 | 30 | 53 | D/Y | — |
| R | 43 | 57 | D/Y | |

[a] Abbreviations are as given for Table 4.

graphic profiles in the monarch. *D. plexippus* reared as larvae on *A. eriocarpa* have a relatively constant pattern of 16–20 cardenolides, defined as the *A. eriocarpa* cardenolide fingerprint profile (Brower et al., 1982). Similarly, *M. galathea* reared on single grass species have constant flavonoid patterns which may be termed the *Lolium perenne* flavonoid fingerprint profile, the *Festuca rubra* flavonoid fingerprint profile, and so on. The flavonoid patterns of *M. galathea* reared on different grass species are so distinct that once the patterns produced in the butterfly by each grass have been determined, it is possible to "fingerprint" individual wild-captured *Melanargia* butterflies to the species of grass ingested by the larvae in the wild, as has been suggested for *D. plexippus*. Indeed, the suggestion that a basic underlying stable system exists controlling the absorption, distribution, excretion, and storage of cardenolides during the physiologically complex growth and metamorphosis of the monarch butterfly (Brower et al., 1982) can also be applied to the sequestration of flavonoids by

the marbled white butterfly. It must, however, be stressed that although the role of cardenolides in the chemical defense of insects against vertebrate predators has been well documented (Reichstein et al., 1968; Brower, 1969; Duffey and Scudder, 1974; Roeske et al., 1976; Brower et al., 1982), there is no evidence to suggest that flavonoids are involved in insect defense.

The poor survival of *M. galathea* in this experiment may be due to high larval mortality not compensated for by the 55 larvae placed on each food plant at the beginning of the experiment, despite the removal of avian predators. Alternatively, differences in the suitability of grass species as larval food plants may have contributed to larval mortality. Factors affecting the suitability of grass species as larval food plants may include the presence or absence of feeding stimulants necessary to elicit feeding after larval diapause; the presence or absence of feeding deterrents, the toughness of the cuticle, the silica content and therefore digestibility of the leaves, and the presence of hairs on the leaf surface which may act as a physical barrier preventing the larvae from feeding.

Since the butterfly flavonoid pattern is dependent on the flavonoid content of the diet, and different *Melanargia* species and varieties possess an identical flavonoid pattern, it appears that the same or very closely related grass species must be utilized as larval food plants by these butterflies throughout their range. If this is so, it may be that the unexplained occurrence of *Melanargia* in locally abundant colonies, frequently confined to a certain field or stretch of ground, is a reflection of the larval food plant species distribution.

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# INDIVIDUAL VARIATION IN THE PHEROMONE OF THE TURNIP MOTH, *Agrotis segetum*[1,2]

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**Abstract**—Female turnip moths (*Agrotis segetum*) from a laboratory culture inbred for more than 30 generations, and the offspring (first and third generation) from field-collected insects were analyzed individually for acetates and alcohols in the pheromone gland. Quantitative analysis of individual components was performed at the subnanogram level by gas chromatography–mass spectrometry (selected ion monitoring). The titer of the pheromone, i.e., the sum of the homologous acetates (*Z*)-5-decenyl acetate, (*Z*)-7-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate was 2.0 ± 0.3 ng in the laboratory culture and 3.2 ± 0.6 ng in the "wild strain." There was no correlation between pheromone titer and female weight. The relative proportion of the pheromone components varied substantially between individuals, but there was no statistically significant difference between the two populations. The percentages of the respective compounds ($\bar{X}$ ± coefficient of variation) were 14.8 ± 127% for Z5-10:OAc, 55.6 ± 32% for Z7-12:OAc, and 29.6 ± 59% for Z9-14:OAc. The pheromone composition varied more in the wild strain than in the laboratory culture. The significance of the pheromone variation to the attraction of males was tested in a field experiment. The ratio of males trapped by the most attractive blend versus the least attractive one was 2.2.

**Key Words**—*Agrotis segetum*, turnip moth, Lepidoptera, Noctuidae, sex pheromone, (*Z*)-5-decenyl acetate, (*Z*)-7-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate, individual variation.

[1] Schiff., Lepidoptera: Noctuidae.
[2] This study was made within the Swedish project "Odour Signals for Control of Pest Insects."



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## INTRODUCTION

In general, female moth sex pheromones have been found to be mixtures of two or more compounds, in some cases required as a precise blend to elicit full sexual response. A pheromone component missing in a synthetic blend or small changes in the ratio between compounds can affect the male behavioral response dramatically (Linn and Roelofs, 1983). In tortricid moths the precisely regulated (*Z*)/(*E*)-11-tetradecenyl acetate ratio also seems to be one of the major mechanisms by which reproductive isolation is maintained among sympatric species (Roelofs and Brown, 1982).

There is not yet enough information available on intraspecific variation in pheromone production to generalize about its relation to mating success. Miller and Roelofs (1980) analyzed almost 400 individual females of the redbanded leafroller, *Argyrotaenia velutinana*, known to use a mixture of (*Z*) and (*E*)-11-tetradecenyl acetate (*Z*11- and *E*11–14:OAc) in its pheromone. The *Z*/*E* ratio was found to be precisely regulated to 91:9, which is close to the ratio found to be maximally attractive to males in the field (Roelofs et al., 1975). The European corn borer (*Ostrinia nubilalis*), a pyralid, also has *Z*11- and *E*11–14:OAc as main components of the pheromone. In nature at least two forms, with opposite ratios between the two isomers, coexist in some areas and, although hybrids are formed, the polymorphism is maintained. One probable mechanism for this is male preference for the pheromone of its own strain (Klun and Maini, 1979; Cardé et al., 1978).

In the turnip moth, *Agrotis segetum*, (Noctuidae), the pheromone consists of at least three homologous monounsaturated acetates: (*Z*)-5-decenyl acetate (*Z*5–10:OAc), (*Z*)-7-dodecenyl acetate (*Z*7–12:OAc), and (*Z*)-9-tetradecenyl acetate (*Z*9–14:OAc) (Löfstedt et al., 1982; Arn et al., 1983). In general, the three acetates are recovered from female abdominal tips in quantities between 0.05 and 2 ng/female. In addition to these compounds, the gland extracts contain at least another 10 compounds chemically related to the behaviorally active pheromone components (Löfstedt et al., 1982).

The field screening tests of Arn et al. (1983) showed that baits with a wide range of ratios between the three homologous acetates were equally attractive to male turnip moths. This suggested to us that the pheromone production in the turnip moth females need not be precisely regulated. In the present study individual variation in pheromone production in two strains of turnip moths was quantified by gas chromatography–mass spectrometry in the selected ion monitoring (SIM) mode. One strain was inbred for more than 30 generations and the other was first- to third-generation offspring from insects collected as pupae in the field. Field-trapping experiments were carried out to obtain a rough estimate of the biological significance of the variation to "mating success," as measured by trapping activity.

## METHODS AND MATERIALS

*Insect Material.* The laboratory strain of *Agrotis segetum*, at the time of this study inbred for more than 30 generations, was characterized by Löfstedt et al. (1982). A "wild strain" was initiated from about 20 wild insects collected as pupae in southern-most Sweden in the autumn of 1981. Insects analyzed from this strain were first- to third-generation offspring of the field-collected specimens. All insects were maintained as described by Löfstedt et al. (1982) on a reversed 16:8-hr light-dark cycle.

*Collection of Pheromone Gland Extracts.* From time of emergence until analysis, insects were kept individually in 250-ml plastic jars and fed a 5% sucrose solution. Ovipositor extracts were prepared from 2 to 6-day-old females. The extraction was performed at the time of maximum calling activity [ca. 4 hr into the scotophase (Löfstedt et al. 1982)] by soaking the tip in 7 $\mu$l heptane with internal standard added. The internal standard was a series of straight-chain acetates including nonyl acetate (9:OAc), undecyl acetate (11:OAc), tridecyl acetate (13:OAc), and pentadecyl acetate (15:OAc); 250 pg of each added per microliter of solvent. The samples were placed in the freezer for about one day, after which the solvent was decanted and transferred to a glass ampoule that was sealed and stored in the dark at $-20°C$ until analysis. Insects were weighed (dry weight, 105°C, 24 hr) after the abdominal tip had been removed.

*Selected Ion Monitoring (SIM).* The insect extract, between 2 and 4 $\mu$l recovered from each female, was injected splitless into a Finnigan 4021 gas chromatograph-mass spectrometer equipped with an Incos data system. The capillary columns used were coated with Superox FA (Supelco Inc.), a polyethylene glycol-type stationary phase. Insects from the laboratory culture were analyzed on a WCOT glass column (62 m $\times$ 0.25-0.30 mm ID) with a film thickness of 0.31 $\mu$m, while a WCOT fused silica column (25 m $\times$ 0.2 mm ID) with a film thickness of 0.37 $\mu$m was used for the wild insect offspring. The helium flow through the columns was 22 cm/sec at 60°C. The injector temperature was 210°C, and the split valve was opened 0.5 min after injection. The column temperature was maintained at 60°C for 5 min following injection, and then heated to 230 at 10°C/min.

The electron energy used was 60 eV (optimized) and the temperature in the ion source 250°C. The separations were monitored by focusing the mass spectrometer on fragments $m/z$ 61 and $m/z$ 82. Mass windows used were $m/z$ 60.6-61.4 and 81.6-82.4, and the scanning times were 0.178 and 0.349 sec for the laboratory and the "wild strain" insects, respectively. The width of the peaks at half height was 3-4 sec, which gave at least eight scans per peak. In the course of the insect extract analyses, the identities of the specific compounds were checked by comparison with retention times of synthetic references.

For quantification, the intensities of $m/z$ 61 and $m/z$ 82 were summarized

TABLE 1. DATA FROM CALIBRATION CURVES FOR SIM ANALYSIS OF MOTH PHEROMONE GLAND CONSTITUENTS

| Compound | Response factor[a] | Coefficient of correlation | LOD (ng)[b] | LOQ (ng)[b] |
|---|---|---|---|---|
| 10:OAc | 0.82 ± 0.05 | 0.997 | 0.03 | 0.09 |
| 12:OAc | 0.94 ± 0.03 | 0.995 | 0.22 | 0.32 |
| 14:OAc | 0.92 ± 0.05 | 0.994 | 0.21 | 0.30 |
| Z5-10:OAc | 0.63 ± 0.06 | 0.988 | 0.09 | 0.21 |
| Z7-12:OAc | 0.91 ± 0.10 | 0.982 | 0.22 | 0.45 |
| Z9-14:OAc | 0.95 ± 0.11 | 0.981 | 0.21 | 0.43 |
| Z5-10:OH | 0.32 ± 0.03 | 0.984 | 0.05 | 0.11 |
| Z7-12:OH | 0.44 ± 0.05 | 0.979 | 0.11 | 0.21 |
| Z9-14:OH | 0.48 ± 0.07 | 0.963 | 0.14 | 0.28 |

[a] 95% confidence interval.
[b] For calculation of LOD (limit of detection) and LOQ (limit of quantification); see Löfstedt and Odham (1984).

in each scan and the areas of the GC-peaks were determined. These areas were compared with the mean value of the areas of the internal standards. The response factors used in these comparisons were based on analyses of a dilution series of synthetic reference compounds with a fixed amount of the internal standard added. The calibration curve for each compound so obtained was based on 16–20 points (see Table 1). The relative abundance of *m/z* 61 and *m/z* 82 varies among the compounds studied (Löfstedt and Odham, 1984; Lanne et al., 1985); therefore, the response factors of the compounds of interest, relative to the saturated acetates used as internal standards, ranged between 0.325 and 0.947.

Limit of detection (LOD = background signal + three times the standard deviation) and limit of quantification (LOQ = background signal + ten times the standard deviation) (Anonymous, 1980) were conservative and calculated as described in Löfstedt and Odham (1984). A low background signal and a high correlation coefficient of the calibration curve result in low values for LOD and LOQ. For the quantification of constituents of the internal standard (9:OAc, 11:OAc, 13:OAc, and 15:OAc) throughout the whole study, the coefficient of variation did not exceed 7% for any of the compounds.

To obtain an overview of the volatile contents of the individual insects, multivariate data analysis was applied according to Wold et al. (1984). The chemical data from each female were treated as points in a $p$-dimensional space, where $p$ is the number of quantified compounds, 10:OAc, Z5-10:OAc, 12:OAc, Z7-12:OAc, Z7-12:OH, Z9-14:OAc, and Z9-14:OH. A log $(x + 0.001)$ trans-

formation of the titers of the seven compounds was performed. With principal component analysis of such a data set, a two-dimensional window into the seven-dimensional space is calculated in such a way that it shows as much as possible of the spread of the data.

*Field Tests.* Field-trapping experiments were carried out in carrot and sugar beet fields in the province of Skåne, southern Sweden. In a first experiment, 13 different blends of 10:OAc, Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc were prepared so as to cover most of the observed range of ratios between the unsaturated compounds. Because of the unclear behavioral function of 10:OAc, the amount of this compound was held constant at 1.25 μg/bait. The other compounds were varied as proportions of a total 24 μg. Stock solutions of the blends were made up in a mixture of pentane and hexane. The blend in 25 μl solvent was applied to a 1-ml polyethylene capsule with 1.5-mm wall thickness (Kartell, Italy). The solvent was allowed to evaporate at room temperature, and the capsule was closed. The baits were usually put in the traps the same day they were made, but were occasionally stored 1–2 days in the freezer (−20°C) prior to use.

The sticky wing traps used (Albany Inc., Needham Heights, Massachusetts) were checked daily, and the baits were renewed. This step was considered to be important as the compounds of the pheromone have quite different volatilities. Four replicate series of traps (blocks) were run for six nights. Within each series, the traps were spaced at least 20 m apart and placed in a row at a right angle to what was presumed to be the predominant wind direction. The distance between series was at least 300 m.

In a second series of experiments, the absolute amount of one of the homologous acetates was varied over two orders of magnitude, whereas the amounts of the remaining two were kept constant. The standard bait used in this experiment was a 1:5:2.5 mixture of Z5–10:OAc, Z7–12:OAc, and Z9–10:OAc. The experiment was performed at two dosages with 0.3 and 3 μg, respectively, of the Z5–10:OAc in the standard bait. Stock solutions of the blends were made up in hexane. The blend in 100 μl solvent was applied to a rubber septum (A.H. Thomas Company, Philadelphia, Pennsylvania). The solvent was evaporated at room temperature, and the dispenser was then stored at −20°C until it was used. The sticky traps used were made in our laboratory. They had a 6-cm opening in all directions, a 20 × 20-cm$^2$ sticky bottom, and a cover of the same size (Löfqvist and Jönsson, unpublished). Five replicate series of traps (blocks) were spaced out and placed as in the first experiment described above.

*Chemicals.* Compounds for the field experiments were at least 99% pure regarding geometrical and positional isomers and the overall chemical purity was more than 98%. 10:OAc was purchased from Schuchardt AG, München, FRG, and Z7–12:OAc and Z9–14:OAc from the Institute for Pesticide Research, Wageningen, The Netherlands. Z5–10:OAc was a gift from Bernt Thelin, Lund University, Sweden.

## RESULTS

*Chemical Identification and Quantification of Pheromone Components and Related Compounds in Gland Extracts.* The SIM analyses were usually selective enough to provide neat tracings of individual tips and the blank showed an essentially smooth baseline (Figure 1A and B). For positive identification of the chromatographic peaks, both the retention index and the ratio of *m/z* 61 to *m/z* 82 had to correspond with those of the synthetic reference compounds. Only occasionally were there reasons to question the identity of a peak (see below for Z9–12:OAc).

The quantification report for each insect was compared to the calculated LOQ values. Most values for Z7–12:OAc and Z9–14:OAc were above the LOQ. For the third behaviorally active monounsaturated acetate, Z5–10:OAc, about half the values were above LOQ. For the remaining compounds (see Table 1), the quantitative reports should be considered approximate as only few values exceeded LOQ. Bar diagrams of the titer of 10:OAc, Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc in the individual females are displayed in Figure 2. Most probably the low amount of the pheromone components did not contribute to the variation, as there was no correlation between the means of the quantified compounds in the two populations and their respective coefficients of variation ($r$ = 0.01, $N$ = 15).

*Comparison of Laboratory-Culture and "Wild-Strain" Insects.* An overview comparison of the two populations revealed a higher spread in the composition of the pheromone gland secretions in the wild strain insects (Figure 3). One extreme female, containing no Z7–12:OAc and no Z9–14:OAc, was excluded from the data set. The amounts of Z7–12:OAc and Z9–14:OAc were not significantly different in the two populations (Table 2). However, the amount of Z5–10:OAc was higher in the wild insects ($\bar{x}$ = 0.35 ng) than in the insects from the laboratory culture ($\bar{x}$ = 0.17 ng). Also the 10:OAc titer was higher in the "wild-strain" insects than in the laboratory strain, but the difference was not significant. Because of the very skewed distribution of the 10:OAc titer in the "wild-strain" population, a nonparametric test (Mann-Whitney $U$ test) was employed for the comparison. The compound earlier assigned as Z9–12:OAc (Löfstedt et al., 1982) was excluded from the comparison of the two strains because of the drifting retention times observed in the "wild-strain" insects. It may be noticed that the relative composition of the gland extracts was very variable. In some females, the amount of single compounds was below the limit of detection.

The sum of the behaviorally active mono-unsaturated acetates (Z5–10:OAc + Z7–12:OAc + Z9–14:OAc) was calculated for each individual moth. The higher average titer in the "wild-strain" insects was not statistically significant (Table 2). The proportions of Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc, respectively, were also calculated (Figure 4A), but no statistically significant dif-

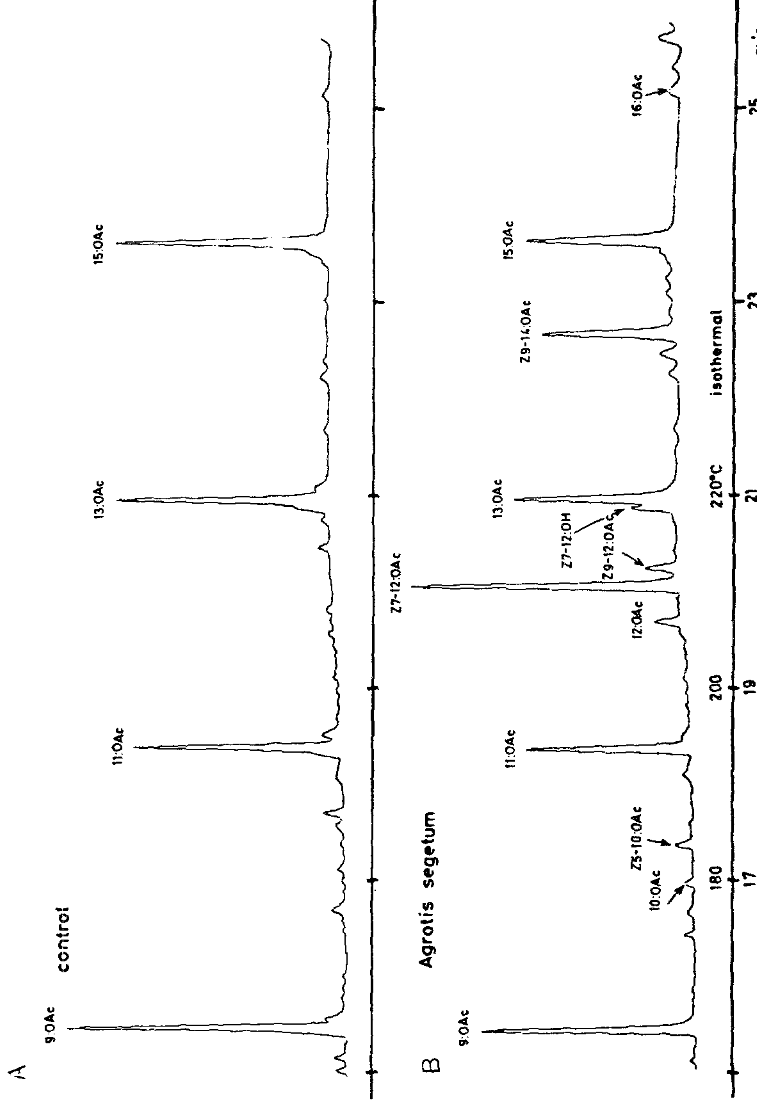



Fig. 1. Reconstructed ion chromatograms ($m/z$ 61 + 82) obtained from GC-MS analyses of a control (A) and an individual *Agrotis segetum* female ovipositor extract (B). The peaks of the internal standards correspond to 1.75 ng each.

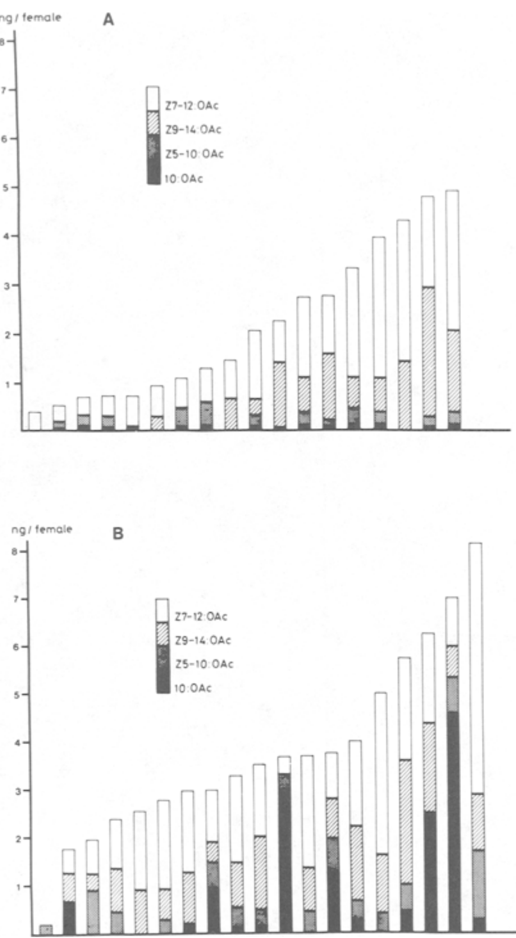



FIG. 2. Absolute amount of 10:OAc, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in ovipositor extracts from individual laboratory-reared strain (A) and wild strain (B) *Agrotis segetum* females. The individuals are arranged in order of increasing pheromone titer.

ferences between the two populations were found (Table 3). Thus the figures from both populations were pooled to calculate the mean proportion of the pheromone components for all females ($N = 38$) and the correlation between the titers of the respective compounds (Table 4). Whereas there was a significant positive correlation between the titers of Z7-12:OAc and Z9-14:OAc, Z5-10:OAc was not significantly correlated to either of Z7-12:OAc or Z9-14:OAc.

The average weight of the "wild-strain" insects ($\bar{X} \pm$ SEM) was significantly higher than that of the laboratory culture, 126 ± 9 and 86 ± 6 mg,

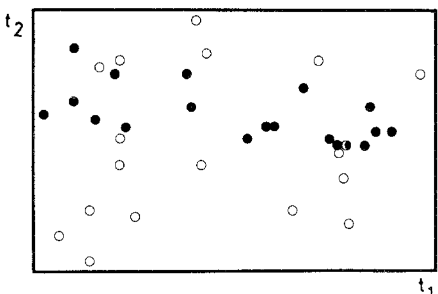



FIG. 3. Principal component analysis of the titer of seven pheromone gland constituents (10:OAc, Z5-10:OAc, 12:OAc, Z7-12:OAc, Z7-12:OH, Z9-14:OAc, and Z9-14:OH) in 37 *Agrotis segetum* females. The 37 points in the seven-dimensional space are projected onto a plane, chosen in such a way that the summed least-squares distances of the 37 points to the plane is minimized. • = laboratory culture, ○ = wild-strain insects. Most of the variation along the $t_2$ axis is due to variation in 10:OAc titer.

TABLE 2. AVERAGE AMOUNT OF PHEROMONE COMPONENTS AND ANALOGS IN EXTRACTS OF INDIVIDUAL *A. segetum* FEMALES FROM LABORATORY CULTURE ($N$ = 18) AND WILD STRAIN OF INSECTS ($N$ = 19)[a]

| Compound | Laboratory culture[b] ($\bar{X} \pm$ SE, ng) | Wild strain[c] ($\bar{X} \pm$ SE, ng) | Statistics (two-sided) | |
|---|---|---|---|---|
| 10:OAc | 0.07 ± 0.01 | 0.74 ± 0.29 | $P$ = 0.43 | Mann-Whitney U test |
| Z5-10:OAc | 0.17 ± 0.03 | 0.35 ± 0.06 | $P$ = 0.02 | Student's $t$ test |
| Z5-10:OH | [d] | 0.20 ± 0.04 | | |
| 12:OAc | 0.16 ± 0.02 | 0.09 ± 0.02 | $P$ = 0.43 | Student's $t$ test |
| Z7-12:OAc | 1.20 ± 0.20 | 1.57 ± 0.26 | $P$ = 0.26 | Student's $t$ test |
| Z7-12:OH | 0.36 ± 0.06 | 0.40 ± 0.04 | $P$ = 0.49 | Student's $t$ test |
| Z9-14:OAc | 0.64 ± 0.16 | 1.32 ± 0.43 | $P$ = 0.15 | Student's $t$ test |
| Z9-14:OH | 0.18 ± 0.02 | 0.08 ± 0.03 | $P$ = 0.02 | Student's $t$ test |
| Sum of behaviorally active acetates (Z5-10:OAc + Z712:OAc + Z9-14:OAc) | 2.01 ± 0.32 | 3.23 ± 0.57 | $P$ = 0.07 | Student's $t$ test |

[a] All values were included, also those below LOQ.
[b] Inbred for more than 30 generations.
[c] First- to third-generation offspring from insects collected in the field.
[d] Not quantified because of interference with other peak.

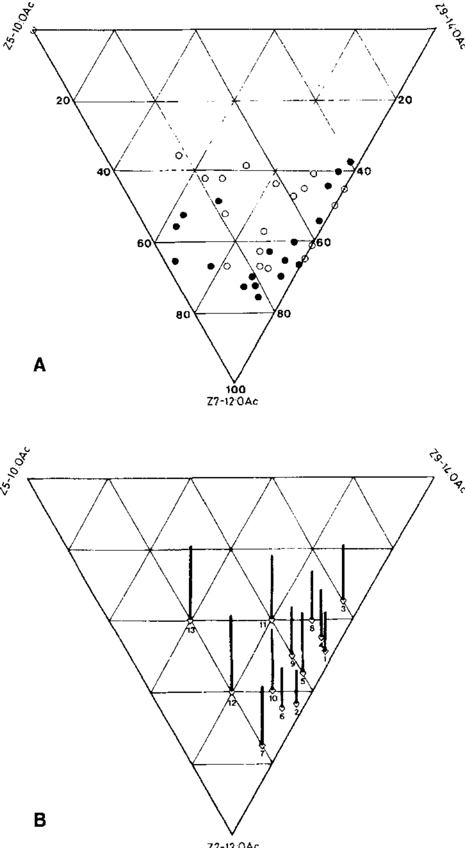



FIG. 4. (A) Relative amounts of Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in ovipositor extracts of individual *Agrotis segetum* females. • = laboratory-reared strain ($N$ = 18) and ○ = wild-strain culture ($N$ = 19). In each corner of the triangle, the relative amount of one compound is 100% and along the opposite side of the triangle the amount of this compound is 0%. As an example, the values for Z7-12:OAc are given in the figure. (B) Mean trap catches ($N$ = 6) of male *A. segetum* in a field experiment with different mixtures of Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc. For bait numbers and statistical data, see Table 5.

respectively ($P$ = 0.001, Student's $t$ test). There was no correlation between the dry weight of all the females ($N$ = 36) and their pheromone titre, i.e., the sum of Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc ($r$ = 0.26, $P$ = 0.12, Pearson).

*Male Response to Pheromone Blends of Individual Females in the Field*
The synthetic lures, approximately covering the observed variation in individual female pheromone composition, differed significantly in attractivity (Fig. 4B and Table 5). More than 1200 males were trapped during the experiment. Catches

TABLE 3. RELATIVE PHEROMONE GLAND TITER (%) OF PHEROMONE COMPONENTS

| Compound | Laboratory strain ($N = 18$) $\bar{X} \pm$ SEM | Wild strain ($N = 19$) $\bar{X} \pm$ SEM | Two-sided $t$ test ($P$ value) | Pooled data ($N = 38$) $\bar{X}$ | Coefficient of variation (%) | Coefficient of variation (arcsin-transf) (%) |
|---|---|---|---|---|---|---|
| Z5-10:OAc | 13 ± 3 | 17 ± 5 | 0.48 | 15 | 127 | 83 |
| Z7-12:OAc | 61 ± 3 | 50 ± 5 | 0.07 | 56 | 32 | 27 |
| Z9-14:OAc | 26 ± 4 | 33 ± 4 | 0.31 | 29 | 59 | 41 |

TABLE 4. CORRELATION BETWEEN TITERS OF PHEROMONE COMPONENTS USING POOLED DATA FROM LABORATORY AND WILD STRAIN ($N = 38$)

| Compounds | Coefficient of correlation (Pearson) | $P$ |
|---|---|---|
| Z5-10:OAc with Z7-12:OAc | 0.14 | 0.39 |
| Z5-10:OAc with Z9-14:OAc | 0.14 | 0.42 |
| Z7-12:OAc with Z9-14:OAc | 0.37 | 0.02 |

TABLE 5. MEAN TRAP CATCHES ($N = 6$) OF MALE *A. segetum* IN FIELD EXPERIMENT WITH DIFFERENT PROPORTIONS OF PHEROMONE COMPONENTS[a]

| Bait No. | Amount in polyethylene cap (μg) | | | | Mean catch[b] |
|---|---|---|---|---|---|
| | 10:OAc | Z5-10:OAc | Z7-12:OAc | Z9-14:OAc | |
| 1 | 1.25 | 0.50 | 12.25 | 12.25 | 14.5 de |
| 2 | 1.25 | 0.50 | 16.00 | 8.50 | 12.7 e |
| 3 | 1.25 | 1.25 | 8.75 | 15.00 | 20.2 abcde |
| 4 | 1.25 | 1.25 | 11.25 | 12.50 | 17.6 bcde |
| 5 | 1.25 | 1.25 | 13.75 | 10.00 | 22.3 abcd |
| 6 | 1.25 | 1.25 | 16.25 | 7.50 | 15.1 cde |
| 7 | 1.25 | 1.25 | 18.75 | 5.00 | 21.7 abcd |
| 8 | 1.25 | 2.50 | 10.00 | 12.50 | 18.2 bcde |
| 9 | 1.25 | 2.50 | 12.50 | 10.00 | 17.9 bcde |
| 10 | 1.25 | 2.50 | 15.00 | 7.50 | 22.7 abc |
| 11 | 1.25 | 5.00 | 10.00 | 10.00 | 23.8 ab |
| 12 | 1.25 | 5.00 | 15.00 | 5.00 | 28.2 a |
| 13 | 1.25 | 10.00 | 10.00 | 5.00 | 27.8 a |

[b]Cf. Figure 5.

[b]Means followed by the same letter are not significantly different by analysis of variance ($\sqrt{X + 0.5}$ transformation) followed by Duncan's multiple range test ($P < 0.05$).

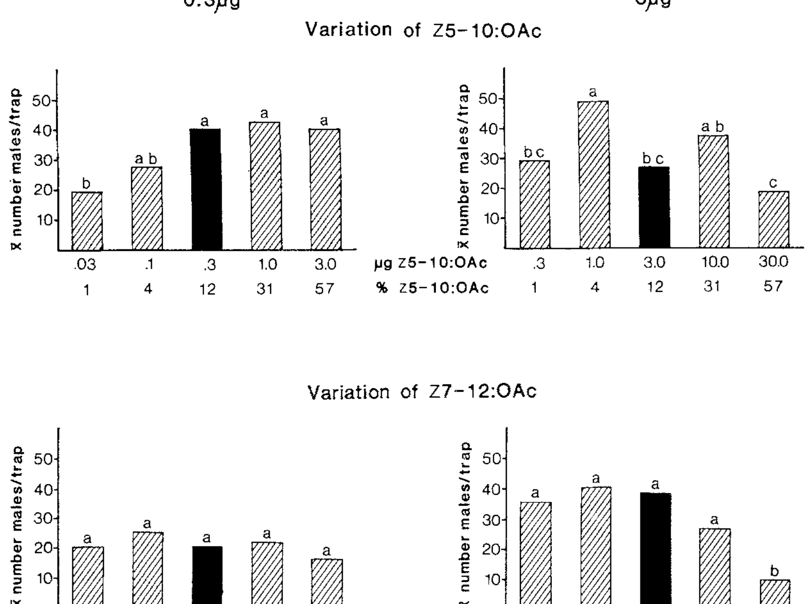
with the synthetic lure that comes closest to the average female pheromone composition (15:66:30), did not differ significantly in attractivity from the most attractive lure (No. 10 vs. 12) (Table 5). There is a positive correlation between the amount of Z5-10:OAc in a bait and its attractivity ($r = 0.76$). In a second experiment, more extreme ratios between the homologous acetates were tested. The amount of one of the unsaturated compounds was varied over two orders of magnitude, while the remaining two were kept at constant level (Figure 5). At the low dose (0.3 $\mu$g), there was a tendency for highest catches with the highest amounts of Z5-10:OAc and Z9-14:OAc, while the amount of Z7-12:OAc did not seem to be critical at all. At the higher dose (3 $\mu$g), highest trap catches were achieved with intermediate amount of all of the compounds. The difference between the most and the least attractive baits was not more than four-fold.

## DISCUSSION

This study shows a considerable variation between individual female turnip moths in the proportions of the pheromone components. The high coefficients of variation (27-83% after arcsin transformation) indicates a pheromone system very different from the redbanded leafroller, for which Miller and Roelofs (1980) found a precisely regulated isomeric ratio with a 9.7% cv. Even though Z7-12:OAc generally was the most abundant pheromone component, there were actually several females where Z5-10:OAc or Z9-14:OAc was the major constituent (Figure 3). Specimens were also found in which one or both of these compounds could not be detected. This is remarkable as both field and flight-tunnel experiments have shown a strong decrease in attractivity when one of the components in the three component mixture is missing (Arn et al., 1983; Löfstedt et al., 1984).

The variation in 10:OAc titer in the wild insects is very striking. Initially we focused on 10:OAc as a gland constituent with possible behavioral activity (Löfstedt et al., 1982). However, parallel to the present investigation of individual variation, detailed behavioral experiments were performed, and they did not reveal any activity of this compound (Löfstedt et al., 1985). Further investigations are needed to reveal the true nature of the large peak with retention time corresponding to that of 10:OAc and the possible behavioral significance of this component.

Shorey and Gaston (1965) found a correlation between pupal weight and subsequent pheromone production, but neither we nor Miller and Roelofs (1980) were able to establish such a correlation between the weight of the imagoes and the pheromone titer. Compared to the wild-strain insects, the body weight of our laboratory insects was significantly lower, but the only significant decrease in a pheromone component in the laboratory strain was observed for Z5-10:OAc. The tendency of a lower pheromone titer in the laboratory insects agrees with



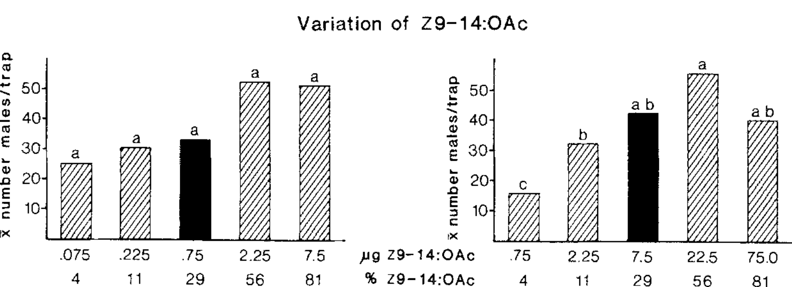




FIG. 5. Mean trap catches ($N = 5$) of male *A. segetum* in two series of field experiments. In each series the amount of one pheromone component was varied over two orders of magnitude, while the remaining two were kept constant. A 1:5:2.5 mixture of Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc was included in each series (= black bar) as a standard. The two dosages are assigned on their content of Z5-10:OAc in the standard bait. The numbers of males trapped are compared within each series by one-way analysis of variance ($\sqrt{x + 0.5}$ transformation) followed by Duncan's multiple-range test. Bars labeled with the same letter indicate means that are not significantly different ($P < 0.05$).

what Miller and Roelofs (1980) found in the redbanded leafroller. Lower pheromone production in laboratory cultures could, of course, be explained as a result of selection in this direction under laboratory conditions, but a mere loss of vigor due to inbreeding and feeding on synthetic diets is an alternative explanation.

We observed a difference in attractivity between our synthetic blends, approximately covering the range of female variation in pheromone production. Theoretically there should be low additive genetic variance in characters (such as sexual attractants) closely related to fitness (Maynard Smith, 1978). A solution to the paradox could be that the female variation documented by us is not genetically determined, contrary to the variation in the redbanded leafroller (Wendell Roelofs, personal communication) and in the European corn borer (Klun and Maini, 1979). The pheromone composition could vary with, for instance, female age or time of day. An alternative explanation would be that the range of variation in attractivity observed in this study is evolutionarily unimportant; males can mate several times and all females that produce at least a minimum amount of the pheromone components will eventually become mated. The ultimate test of these hypotheses has to wait until we know the range of ratios actually released by calling females, and these can be precisely reproduced with synthetic dispensers.

We assume that pheromones produced by Lepidoptera in intersegmental glands are given off by passive diffusion. Blend components with different chain lengths (or different functional moieties) would then evaporate at slightly different rates from the gland surface at different environmental temperatures, according to differences in the temperature dependence of their vapor pressures. Hence the composition of the effluvium from a calling female would vary with temperature. Cardé and Baker (1984) have suggested that this is the raison d'être of several divergent blends in moths, precluding the use of such compounds in very precise ratios. However, this is not a sufficient explanation for divergent pheromone blends even in the case of the turnip moth, although this species uses pheromone components of three different chain lengths. By use of Raoult's law and vapor pressures extrapolated from Olsson et al. (1983), we calculated the composition of the vapor phase corresponding to a certain female pheromone (gland surface) composition (Table 6). It is obvious that even extreme temperature changes will only cause small changes in the vapor phase composition. For example, a temperature shift from 5°C to 20°C or from 20°C to 30°C will, at most, shift the pheromone composition 3%. This is far less than the range of female-produced ratios and the range of blends attractive to males actually observed in this study. We think that there is an evolutionary cost associated with the production of precise pheromone blends. A precisely regulated pheromone will evolve only when there is a strong selection in favor of species specificity achieved by this means. In the turnip moth, the three homologous unsaturated

TABLE 6. THEORETICAL COMPOSITION OF VAPOR PHASE[a] OVER LIQUID MIXTURE OF *A. segetum* PHEROMONE COMPONENTS AT DIFFERENT TEMPERATURES

| | Weight (%) | | | |
|---|---|---|---|---|
| | In liquid phase | In vapor phase | | |
| | | 5°C | 20°C | 30°C |
| Z5-10:OAc | 11.7 | 64.6 | 61.6 | 59.7 |
| Z7-12:OAc | 58.8 | 33.6 | 35.8 | 37.2 |
| Z9-14:OAc | 29.4 | 1.8 | 2.5 | 3.1 |

[a] Calculated from the composition of the liquid phase using Raoult's law and extrapolated *P* values from Olsson et al. (1983).

acetates might give enough specificity on a qualitative basis and a regulation of ratios is therefore not critical.

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# ESTER COMPONENTS OF AGGREGATION PHEROMONE OF *Drosophila virilis* (DIPTERA: DROSOPHILIDAE)

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**Abstract**—The male-produced aggregation pheromone of *Drosophila virilis* was found to contain five ester components, in addition to a previously identified hydrocarbon, (*Z*)-10-heneicosene (Z10–21). The five esters were: the methyl, ethyl, and 1-methylethyl (isopropyl) esters of 2-methyl-(*E*)-2-butenoic (tiglic) acid and the methyl and ethyl esters of hexanoic acid. The esters were not detected in females. Each ester was active by itself in laboratory bioassay tests, and each increased the number of flies responding to Z10–21 ca. 4–5 times. In comparisons among the five esters at 10 ng per compound, ethyl tiglate was the most active, and methyl tiglate, the least. No mixture of esters was found to be significantly more active than ethyl tiglate alone. In a dose-response study, bioassay activity increased with dose for both ethyl tiglate and Z10–21. Newly emerged males did not have detectable levels of the esters. All five esters increased as sexual maturity was approached. Ethyl tiglate and ethyl hexanoate were the most abundant in mature males, usually over 15 ng per individual. Ratios among the esters were variable. Male flies also contained an as yet unidentified attractant(s) still more polar than the esters, which was synergistic with the esters and hydrocarbon. Food odors also synergized the synthetic compounds.

**Key Words**—*Drosophila virilis,* Diptera, Drosophilidae, aggregation, pheromone, 2-methyl-(*E*)-2-butenoic acid, hexanoic acid, ester.

## INTRODUCTION

*Drosophila virilis* Sturtevant is a cosmopolitan species of fruit fly. Bartelt and Jackson (1984) showed that sexually mature males of *D. virilis* produce an aggregation pheromone to which flies of both sexes and any age are attracted in a



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laboratory bioassay. The pheromone was shown to consist of a hydrocarbon, (Z)-10-heneicosene (Z10–21), and at least one additional component with the polarity of an ester. This more polar portion of the pheromone has been studied further, and we report here the isolation, identification, and synthesis of five ester pheromone components from *D. virilis* males. The bioassay properties of these esters, alone and in combination with Z10–21, and their production with respect to age and sex are also discussed.

## METHODS AND MATERIALS

*Flies and Bioassays.* The *D. virilis* culture was obtained from the University of Texas at Austin. The flies were reared in 1-liter jars on Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply Co., Burlington, North Carolina) under existing temperature and light conditions. Flies to be extracted were sexed at 0–1 days of age while immobilized by cold or Fly-Nap (Carolina Biological). Unless otherwise indicated, these were fed until 7–14 days old in 3.5 × 10-cm vials with 100–200 flies per vial.

Flies for bioassays were removed from the rearing jars when 0–6 days old, starved overnight in the bioassay olfactometer, and tested the following morning. The bioassay procedure and apparatus were fully described by Bartelt and Jackson (1984). Briefly, the wind-tunnel olfactometer was large enough to allow free flight and was stocked with ca. 1000 flies. A pheromone sample, in solvent, was applied to a filter paper strip inserted around the lip of a glass vial. Two such treated vials, to be compared, were placed on the floor of the olfactometer in the upwind end. Responses included upwind casting flight, landing on the vial, and crawling down inside. Each vial contained a drop of water, which was not itself attractive but which caused the flies that entered the vial to remain throughout the test. Tests lasted 3 min, after which the vials were capped and the flies counted. As many as 50 tests could be run in the course of a day after stocking the olfactometer once. Because the effect of the ester fraction was easiest to see in conjunction with Z10–21 (Bartelt and Jackson, 1984), this hydrocarbon was added to most test vials and control vials.

Each bioassay experiment used the balanced incomplete block design, in which the treatments were tested in pairs in all possible combinations. The counts of flies were transformed to the $\log(n + 1)$ scale to stabilize variance and the analysis conducted according to Yates (1940). Other details of experiments are given with results.

*Extraction, Purification, and Identification of Pheromone Components.* Flies were extracted by soaking in hexane for 24 hr at room temperature. The crude extract was separated by polarity on an open column of silicic acid (Bio-Sil A, Bio-Rad Laboratories, Richmond, California) as described by Bartelt and Jackson (1984). The ester fraction, eluted with 7.5% ether in hexane, was sep-

arated further by HPLC using a size exclusion column (PLGel 10 μm, 50 Å, 30 cm × 7.7 mm ID, Polymer Laboratories, Shropshire, U.K.). This column separated compounds primarily on the basis of molecular size, with the larger ones eluting first. The elution solvent was hexane, delivered by a Waters Associates M-6000A pump at 2 ml/min, and the effluent was monitored with a Waters Associates 401 differential refractometer detector. Samples for HPLC separations varied from 100 to 1000 fly equivalents. Further HPLC separations on the active size-exclusion fractions were carried out on a silicic acid column (Adsorbosphere Silica 5 μm, 250 × 4.6 mm ID, Alltech Assoc., Deerfield, Illinois), eluted with 5% ether in hexane at 1 ml/min.

The collected fractions were tested by bioassay and were examined by gas–liquid chromatography (GLC). A Varian 3700 gas chromatograph fitted with a Durabond DB-225 or Durabond DB-1 capillary column (30 m × 0.25 mm ID, J. & W. Scientific, Rancho Cordova, California), was used for all analyses of esters. The carrier gas was He at 50 cm/sec, and various temperature programs were used. All samples were concentrated under nitrogen prior to GLC analysis.

Electron impact mass spectra of the isolated active compounds were obtained on a VG MM16 or VG 7070 mass spectrometer, using the DB-225 capillary GLC column for introduction of samples. The ionization energy was 70 eV. Various GLC temperature programs were used. High-resolution spectra of some samples were also obtained on the VG 7070.

*Synthetic Tiglate and Hexanoate Esters.* Tiglic acid (Aldrich Chemical Co., Milwaukee, Wisconsin) and hexanoic acid were converted to acyl chlorides with thionyl chloride, and these were allowed to react with the appropriate alcohols (methanol, ethanol, 1-propanol, or 2-propanol) to form esters. After purification by column chromatography on silicic acid and HPLC, the tiglate esters were at least 98% pure and the hexanoate esters 95% pure by capillary GLC. They were diluted to 1 ng/μl with hexane for use in most bioassay experiments. Mass spectra of the synthetic esters were obtained.

*Measurements of Tiglate and Hexanoate Esters in Groups of Flies.* In an experiment to determine the amounts of the pheromone esters as a function of age, groups of ca. 100 flies, sexed within 24 hr of emergence, were aged in 1-liter jars with food medium, and extracted at the appropriate age. There were always two replications for each age. All of the flies for this experiment were obtained from the same culture jars over a three-day period.

The groups of flies were extracted by soaking for 24 hr in hexane at room temperature, exact counts being made after extraction. After purification by column chromatography on silicic acid and size-exclusion HPLC (the effluent 13–18 ml after injection being collected), the esters were analyzed by GLC on the DB-225 column (50°C for 5 min then 10°/min to 100°). Propyl tiglate (500 ng) was added to the ester fraction as an internal standard. The hydrocarbon fraction from the column-chromatography step was analyzed for Z10–21 by GLC on a 2 m × 2 mm ID glass column packed with 3% Dexsil 300 (190–300°C at 10°/min). 7-Octadecene was used as the internal standard.

## RESULTS

*Isolation of Pheromone Components.* The attractiveness of the 7.5% ether–hexane fraction from mature males was readily lost when the sample was evaporated to dryness under a stream of nitrogen, suggesting that the pheromone was quite volatile. The size-exclusion HPLC column was employed for the next purification step because very small components could be readily separated from those of high molecular weight without the need to trap volatiles, as with preparative GLC. Table 1A shows examples of bioassay results for one set of HPLC fractions. The 15- to 16-ml fraction caused a 10-fold increase in response over the control, and the other fractions between 13 and 18 ml also showed strong evidence of attractiveness. Similar sets of HPLC fractions were collected for four other male extracts and gave comparable bioassay results. Ester standards eluted from this column in bands about 1 ml broad, and since no single active compound should appear in more than two consecutive 1-ml fractions, there was evidence for more than one active compound. The 15- to 16-ml fraction from the size-exclusion column was purified further by HPLC on silicic acid (Table 1B). Here the activity was primarily in fractions 5–6 ml after injection. The

TABLE 1. PURIFICATION OF 7.5% ETHER–HEXANE BIO-SIL FRACTION BY HPLC[a]

| A. Size exclusion of 7.5% ether–hexane Bio-Sil fraction | | B. Separation of 15 to 16-ml size-exclusion fraction on silicic acid | |
|---|---|---|---|
| Fraction (ml after injection) | Bioassay (control)[b] (flies per test) | Fraction (ml after injection) | Bioassay (control) (flies per test) |
| 9–10 | 1.5 (5.5) | 3.0–4.0 | 8.6 (5.0) |
| 10–11 | 3.5 (1.5) | 4.0–4.5 | 8.6 (3.6) |
| 11–12 | 5.0 (2.5) | 4.5–5.0 | 7.4 (3.4) |
| 12–13 | 4.5 (2.5) | 5.0–5.5 | 25.0 (2.4) |
| 13–14 | 17.3 (2.0) | 5.5–6.0 | 29.6 (4.2) |
| 14–15 | 10.3 (2.8) | 6.0–6.5 | 5.2 (3.0) |
| 15–16 | 29.4 (2.8) | 6.5–7.0 | 4.6 (3.6) |
| 16–17 | 10.3 (3.3) | 7.0–8.0 | 4.0 (3.6) |
| 17–18 | 8.7 (1.0) | 8.0–9.0 | 5.0 (3.3) |
| Parent | | 9.0–10.0 | 4.0 (4.0) |
| Bio-Sil | 20.0 (3.0) | 10.0–11.0 | 7.3 (4.0) |
| fraction | | 11.0–15.0 | 2.8 (3.0) |

[a]Bioassay results for size exclusion fractions are shown in (A). Results for further purification of the 15-16 ml size exclusion fraction on silicic acid are shown in (B). The void volumes of the size exclusion and silicic acid columns were 9 and 3 ml, respectively.

[b]Bioassay scores are mean catches of flies per 3-min test period. The HPLC fractions were used at ca. 2 male equivalents per test. Male hydrocarbons (0.5 fly equivalents) were added to both the test and the control vials.

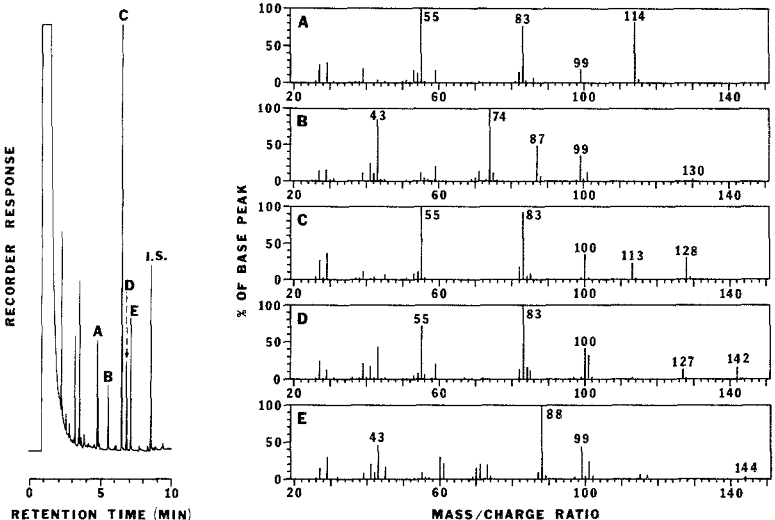


FIG. 1. At left: gas chromatogram of active region from size-exclusion HPLC (13–18 ml). Peaks A–E occurred only in males. Peaks to the left of A were solvent-related and occurred in all samples. I.S. is the internal standard (propyl tiglate). GC conditions: DB-225, 50°C for 5 min then 10°/min to 100°. At right: mass spectra for these GC peaks.

other active size-exclusion fractions were similarly run on silicic acid and bioassayed, and in each case the activity was retained as above, indicating the active compounds were very similar in polarity.

The gas chromatogram of the 13- to 18-ml region of the size-exclusion column from one group of males is shown in Figure 1. At least one of the five lettered peaks was present in each active size-exclusion fraction, with peak A eluting 16–18 ml after injection; peak B, 13–15 ml; peak C, 14–16 ml; and peaks D and E, 13–14 ml. The lettered GLC peaks were also detected in the active silicic acid HPLC fractions, but they were never detected in extracts of females. Thus the five compounds were believed to be pheromone components.

*Identification.* The mass spectrum of GLC peak C in Figure 1 indicated a molecular weight of 128. By the high-resolution mass spectrum, this corresponded to the formula, $C_7H_{12}O_2$, which has two degrees of unsaturation. Because of its polarity, the functional group was believed to be an ester. The *m/e* 83 and 100 peaks, corresponding to the loss of $-OC_2H_5$ and $C_2H_4$, respectively, suggested an ethyl ester. The large *m/e* 113 peak indicated ready loss of a methyl group. The mass spectrum was compared to those of the ethyl esters of branched, unsaturated acids and agreed very well with that obtained by Thomas and Willhalm (1976) for ethyl 2-methyl-(*E*)-2-butenoate (ethyl tiglate). These authors noted that only the *E* isomer has the 113 peak in the mass spectrum, and they also noted that for the *E* isomer, the ratio of *m/e* 83 and 82 peaks is ca. 7 : 1 whereas for the *Z* isomer, the ratio is ca. 2 : 1. With respect to both these fea-

tures, the mass spectrum of the fly-derived ester corresponded well to the *E* configuration.

Thomas and Willhalm (1976) also presented mass spectral data for the methyl, propyl, and isopropyl esters of tiglic acid. The mass spectrum of GLC peak A in Figure 1 was in excellent agreement with methyl tiglate, and peak D, with propyl or isopropyl tiglate. Again, the ratios of *m/e* 83 and 82 peaks indicated the *E* configuration. Since propyl and isopropyl tiglates have only subtly different mass spectra, both synthetic esters were prepared, but only isopropyl tiglate had the same GLC retention as peak D, on both the DB-225 and DB-1 columns. As further evidence for these structures, the GLC retentions of peaks A and C were identical to synthetic methyl and ethyl tiglate, respectively, on both DB-225 and DB-1. Since the *E* and *Z* esters are separable by GLC (Thomas and Willhalm, 1976), the GLC data further supported the *E* configuration of the fly-derived esters. Finally, the mass spectra of the synthetic and fly-derived compounds were in agreement for all tiglate esters.

The mass spectra of GLC peaks B and E indicated molecular weights of 130 and 144, respectively. The intense peaks at 74 and 88 suggested methyl and ethyl esters, respectively, of some saturated acid. Comparison to published spectra (Heller and Milne, 1978) suggested the unknown compounds could be esters of hexanoic acid or of 3- or 4-methylpentanoic acid. The methyl and ethyl esters of the straight-chain (hexanoic) acid were synthesized, and their GLC retention times (DB-225 and DB-1) and mass spectra agreed with those of the fly-derived compounds. (The branched esters would have eluted earlier than the straight-chain esters from the GLC columns.) Thus the five esters derived from *D. virilis* males, in order of elution from the DB-225 column, were methyl tiglate, methyl hexanoate, ethyl tiglate, isopropyl tiglate, and ethyl hexanoate (Figure 2).

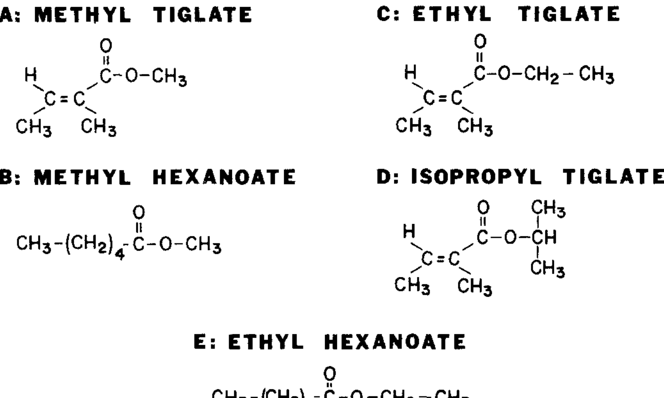


FIG. 2. Structures of the five active esters. (Letters correspond to the peak labels and mass spectra in Figure 1).

TABLE 2. ACTIVITY OF INDIVIDUAL ESTERS WITH AND WITHOUT Z10-21

| | Mean bioassay catch[a] | | | | |
|---|---|---|---|---|---|
| Ester | Ester[b] | Control | Ester + Z10-21 | Z10-21 | *N* |
| Methyl tiglate | 3.5 | 0.4 | 12.0 | 3.4 | 30 |
| Ethyl tiglate | 3.2 | 1.0 | 17.8 | 4.2 | 48 |
| Isopropyl tiglate | 2.0 | 0.5 | 7.4 | 1.8 | 24 |
| Methyl hexanoate | 2.9 | 0.4 | 12.8 | 3.3 | 24 |
| Ethyl hexanoate | 2.3 | 0.8 | 11.4 | 3.0 | 30 |
| Propyl tiglate | 3.9 | 0.1 | 16.6 | 3.0 | 12 |

[a]Each ester differed significantly from the control ($P < 0.001$), and each ester significantly increased the activity of Z10-21 ($P \ll 0.001$).
[b]Each ester was used at 10 ng/test. Z10-21 was used at 1.3 μg/test.

*Bioassay of Synthetic Esters.* As seen in Table 2, each synthetic ester was significantly more attractive than controls in the bioassay. (Each was used at 10 ng, an amount in the range of ca. 0.2-10 mature male equivalents, depending on the ester). In addition, each ester greatly increased the response toward Z10-21 in much the same way as the crude ester fraction from male *D. virilis* had done. At 10 ng, each ester was similar in activity to 1.3 μg (ca. 1/3 mature male equivalent) of Z10-21. Interestingly, propyl tiglate, which was not detected in the flies, was also quite active in these assays.

To determine the relative activity of the identified esters, the five individual compounds and a mixture of all five (in the proportions found in one group of male flies) were compared in pairs in all possible combinations (10 ng of ester per test). Each was tested in combination with Z10-21, and Z10-21 by itself was the "control" in this experiment (Table 3). As in the previous study, each of the esters caused a significant increase in the activity of Z10-21, as did the mixture of all five esters. The most abundant ester in males, ethyl tiglate, tended to be the most active, and methyl tiglate was significantly less active than the others, but differences in activity among the esters were generally subtle. There was no indication that combinations of esters would be strongly preferred over ethyl tiglate.

Both sexes responded about equally throughout this research, regardless of whether the treatments were fly-derived or synthetic, in agreement with earlier findings (Bartelt and Jackson, 1984). Thus all data presented were totaled over sexes.

*Amount of Ester vs. Age.* Males and females between 0 and 15 days of age were analyzed for the pheromone esters and Z10-21. With 100-fly samples, the esters could be detected at 100 pg/fly. Neither the esters nor the hydrocarbon were detected in females of any age. The data for the males are shown in Table

TABLE 3. COMPARISONS AMONG TIGLATE AND HEXANOATE ESTERS (BALANCED INCOMPLETE BLOCK EXPERIMENT)

| Test material[a] | Mean bioassay catch[b] ($N = 24$) |
|---|---|
| Methyl tiglate + Z10-21 | 5.5b |
| Ethyl tiglate + Z10-21 | 13.7de |
| Isopropyl tiglate + Z10-21 | 9.8cd |
| Methyl hexanoate + Z10-21 | 12.6cde |
| Ethyl hexanoate + Z10-21 | 8.9c |
| Mixture[c] + Z10-21 | 16.1e |
| Z10-21 | 2.4a |

[a] All esters used at 10 ng/test; Z10-21 used at 1.3 μg/test.
[b] Means followed by the same letter not significantly different in the log ($n + 1$) scale (LSD 0.05).
[c] 10 ng of mixture of esters with proportions as found in one group of males: 10% methyl tiglate, 47% ethyl tiglate, 18% isopropyl tiglate, 13% methyl hexanoate, and 10% ethyl hexanoate.

4. Newly emerged flies had no detectable tiglates or hexanoates. Ethyl hexanoate was the first ester to reach 1 ng/fly, at 1-2 days of age, followed by ethyl tiglate at 4-5 days. Both the ethyl tiglate and ethyl hexanoate continued to increase with age, and males over 7 days old usually had 15+ ng of each of these esters.

The ethyl tiglate closely paralleled Z10-21 in its time of appearance, whereas ethyl hexanoate preceded it by several days. These flies produced greater amounts of Z10-21 and did so at an earlier age than reported in the previous

TABLE 4. ANALYSIS FOR TIGLATE AND HEXANOATE ESTERS AND Z10-21 IN MALE *D. virilis* BY AGE

| | Amount of compound (ng/male) | | | | | |
|---|---|---|---|---|---|---|
| Age (days) | Methyl tiglate | Ethyl tiglate | Isopropyl tiglate | Methyl hexanoate | Ethyl hexanoate | Z10-21 |
| 0-1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0 |
| 1-2 | 0.0 | 0.0 | 0.0 | 0.1 | 1.0 | 0 |
| 2-3 | 0.0 | 0.3 | 0.0 | 0.2 | 6.0 | 20 |
| 3-4 | 0.1 | 0.1 | 0.0 | 0.2 | 2.8 | 0 |
| 4-5 | 0.2 | 4.9 | 0.0 | 0.2 | 3.2 | 1430 |
| 5-6 | 0.1 | 9.1 | 0.0 | 0.1 | 5.5 | 1920 |
| 6-7 | 0.0 | 14.5 | 0.0 | 0.1 | 8.2 | 2980 |
| 7-8 | 0.7 | 18.9 | 7.6 | 0.7 | 18.4 | 3570 |
| 8-9 | 1.3 | 21.7 | 2.0 | 1.3 | 19.6 | 4760 |
| 10-11 | 0.4 | 44.8 | 1.7 | 0.4 | 15.5 | 2630 |
| 15-16 | 0.5 | 18.2 | 0.5 | 0.2 | 11.2 | 1580 |

paper, probably because of warmer temperatures and better rearing conditions. Nevertheless, the appearance of ethyl hexanoate before Z10–21 may explain the attractiveness of male flies before they reached sexual maturity (Bartelt and Jackson, 1984).

The other three esters were far less abundant. These did not approach 1 ng/fly until 7–8 days of age. The mean 7.6 ng/fly for isopropyl tiglate at 7–8 days was due largely to one group of males (the amounts for the two replicates being 14.8 and 0.5), indicating that great variability in the proportions of esters was possible.

*Dose Response to Ethyl Tiglate and Z10–21.* Since ethyl tiglate was the most abundant ester and was as active as any mixture of esters tested, this ester was used with Z10–21 for a dose–response study (Table 5). To allow for day-to-day variability in bioassay results in this large experiment, each dose trial was tested against a standard mixture of ethyl tiglate and Z10–21 (10 ng and 1.3 $\mu$g, respectively, representing ca. 1/3 male equivalent) and against a hexane control. An index of activity for the trial dose was computed as:

$$\text{Index} = \frac{(\text{test dose} - \text{control})}{(\text{standard dose} - \text{control})} \times 100$$

This index expresses the activity of the test dose as a percentage of the standard dose, corrected for controls. Although absolute catches varied from day to day, ratios remained relatively stable, so the indices from different days could be

TABLE 5. INDEX OF ACTIVITY[a] FOR VARIOUS DOSES OF ETHYL TIGLATE AND Z10–21

| Amount of ethyl tiglate (ng) | Amount of Z10–21 ($\mu$g) | | | |
|---|---|---|---|---|
| | 0.0 | 0.13 | 1.3 | 13 |
| 0.0 | 0[b] | 2 | 27 | 57 |
| | — | (1.2,49.5,0.5) | (4.6,13.6,1.3) | (7.2,11.8,1.0) |
| 1.0 | 9 | 21 | 42 | 126 |
| | (2.1,21.7,0.3) | (4.7,19.4,0.7) | (8.0,18.0,0.7) | (10.4,8.3,0.3) |
| 10 | 10 | 34 | 100[c] | 184 |
| | (1.8,14.2,0.4) | (13.9,38.9,0.8) | — | (14.5,8.0,0.3) |
| 100 | 27 | 27 | 106 | 216 |
| | (3.1,9.9,0.6) | (6.0,19.9,0.9) | (19.9,17.8,2.8) | (15.9,7.8,0.9) |

[a] Index of activity = (test dose − control)/(standard dose − control) × 100. Each index was based on a paired comparison experiment among the test dose, the standard dose, and controls, with 12 replications per treatment. The means for these treatments, respectively, are shown in parentheses. The standard dose was 10 ng ethyl tiglate + 1.3 $\mu$g Z10–21.

[b] Is zero by definition.

[c] Is 100 by definition.

reasonably compared. Each index was based on a paired comparison study with 12 replications per treatment.

Each component, by itself, was significantly more active than the control, even at the lowest levels tested. Increasing either component always increased the activity, except with 100 ng ethyl tiglate and 0.13 μg Z10–21, probably a statistical fluctuation. We found no optimal combination, so that further increases in either component would lower the index.

In another experiment, ethyl tiglate was active at even 100 pg, causing a significant ($P < 0.01$) increase in the activity of 1.3 μg of Z10–21. The means ($N = 12$) for ethyl tiglate + Z10–21, Z10–21, and controls were 21.0, 12.1, and 1.0, respectively.

*Other Attractants* An experiment was conducted to determine how well Z10–21 and the five synthetic esters accounted for the activity of the crude extract of male *D. virilis*. An aliquot of the crude extract was analyzed and a mixture of synthetic compounds prepared, so that for each component the crude extract and synthetic mixture were comparable. The bioassay results are shown in Table 6. The crude extract was significantly more active than the synthetic mixture. However, when the crude extract was applied to a column of silicic acid and eluted with 10% ether in hexane, the effluent (containing the hydrocarbons and esters) was not significantly different from the synthetic mixture in activity. This result was corroborated by an additional experiment, in which this less polar fraction of the crude extract was tested against only the synthetic mixture and controls; the means were 8.7, 10.1, and 0.1, respectively ($N = 8$, means for the two pheromone samples not different, $P > 0.4$). The more polar portion of the crude extract (subsequently eluted from the column with 10% methanol in chloroform) was also active. When the polar and nonpolar fractions

TABLE 6. COMPARISON OF MALE-DERIVED AND SYNTHETIC PHEROMONE COMPONENTS

| Treatment | Mean bioassay catch[a] ($N = 8$) |
|---|---|
| Z10–21 + ester mix[b] | 8.0ab |
| Crude extract[c] | 27.9c |
| Nonpolar portion of extract[d] | 11.2b |
| Polar portion of extract[e] | 6.4a |
| Mix of nonpolar and polar portions | 31.2c |

[a] Means followed by the same letter not significantly different (LSD, 0.05).
[b] Synthetic pheromone components mixed as measured in the "crude extract" and used at a comparable dose. Amounts per test were: 1.6 ng methyl tiglate, 49. ng ethyl tiglate, 2.0 ng isopropyl tiglate, 1.6 ng methyl hexanoate, 16. ng ethyl hexanoate, and 4700 ng Z10–21.
[c] Used at 2 male equivalents/test.
[d] Eluted from Bio-Sil with 10% ether in hexane, and used at 2 male equivalents/test.
[e] Eluted from Bio-Sil with 10% MeOH in $CHCl_3$, after collecting the nonpolar fraction. Used at 2 male equivalents/test.

TABLE 7. COMPARISON OF FOOD AND PHEROMONE AS ATTRACTANTS

| Treatment | Mean bioassay catch[a] ($N$ = 24) |
|---|---|
| Z10-21 + ethyl tiglate[b] | 23.4c |
| Food[c] | 3.2b |
| Z10-21 + ethyl tiglate + food | 44.8d |
| Control | 0.6a |

[a] All means significantly different at the 0.05 level (LSD).
[b] Z10-21 was used at 1.3 μg/test and ethyl tiglate at 10 ng/test.
[c] "Food" was fermented rearing medium, placed in the bottom of the vial.

were combined, the mixture was comparable in activity to the crude extract. Thus, at least under some conditions, there were additional, more polar compounds in the male *D. virilis* which were coattractants of the pheromone components so far identified. But there appear to be no significant, additional attractants in male flies with the polarity of esters or hydrocarbons.

Since aggregation and feeding are believed closely tied in this species (Bartelt and Jackson, 1984), a study comparing the synthetic pheromone and fermented food was conducted (Table 7). In this experiment, which included three groups of bioassay flies, the food was only slightly active by itself, yet it nearly doubled the number of flies responding to the pheromone. A combination of food and pheromone scents may be of great ecological importance to the species.

## DISCUSSION

This report continues to describe the aggregation pheromone system in *D. virilis* that was introduced in an earlier paper (Bartelt and Jackson, 1984). That paper demonstrated the existence of a pheromonally active hydrocarbon and at least one pheromone component with the polarity of an ester. As shown here, not one but five active esters have been isolated from that fraction. Each of these esters synergized the active hydrocarbon identified in the earlier work, in much the same way that the crude ester fraction did. The esters, like the hydrocarbon, are produced only by male flies, and their abundance increases dramatically as sexual maturity is approached. Because the ester and hydrocarbon components appear only in males at about the same time and because they are synergistic in bioassay, we believe they would operate as a unit under field conditions. Yet this pheromone, as a whole, is unusual in that the components differ so widely in molecular weight. The smallest ester has 6 carbon atoms whereas the hydrocarbon has 21. The components also differ greatly in amount present. The most abundant ester, ethyl tiglate, represents only about 1% of the amount of Z10-21.

Exactly how the five distinct esters function is unclear. Although certain esters were somewhat more attractive than others (e.g., ethyl vs. methyl tiglate), the bioassay properties of all five were qualitatively similar. Mixtures of the esters, as found in groups of males, were not greatly superior to individual esters. Because methyl tiglate, methyl hexanoate, and isopropyl tiglate were found in relatively minor amounts, these may be of limited importance as pheromone components. The flies may not be able to discriminate among all the esters, since propyl tiglate was quite active even though it was never detected in *D. virilis*. Perhaps a more natural bioassay system will be required to resolve the functions of the various esters, especially between the tiglates and hexanoates.

Many questions remain about the pheromone system in this species. The effects of environmental parameters on pheromone production and responsiveness to pheromones need to be examined, especially since the adults are so long-lived. The biosynthesis of these esters would also be of interest. Could the alcohol moieties be derived from the fermenting food medium? Finally, there is evidence that attractants still more polar than esters can be extracted from the flies, and these need to be identified. The aggregation phenomenon in *Drosophila* appears to be extremely complicated, but probably involves a large number of chemicals, some produced by the flies and some by the food medium.

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# BEHAVIORAL RESPONSES OF MALE TURNIP MOTHS, *Agrotis segetum*,[1] TO SEX PHEROMONE IN A FLIGHT TUNNEL AND IN THE FIELD[2]

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**Abstract**—The response of individual male turnip moths *Agrotis segetum* was observed in a sustained flight tunnel to a mixture of decyl acetate, (*Z*)-5-decenyl acetate, (*Z*)-7-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate in proportions similar to those found in gland extracts from virgin females (0.6 : 1 : 5 : 2.5). Lures containing 3–30 μg (*Z*)-5-decenyl acetate proved to be maximally attractive, with approximately 60% of the males completing all behavioral steps from activation to copulation efforts. A 300-μg dosage caused significant arrestment of upwind flight. Peak response to synthetics, however, was significantly lower than to female glands. Omitting decyl acetate from the blend did not affect the activity, while omission of any of the three monounsaturated acetates caused a dramatic decrease in response. In the field maximum trap catches were achieved with 1- to 30-μg lures. The subtractive assay carried out in the field confirmed the neutrality of decyl acetate and the importance of the three monoenes. Adding 1% of (*Z*)-8-dodecenyl acetate (earlier reported as an "inhibitor") to the four-component mixture decreased the trap catch to about 50%, and increasing the amount of (*Z*)-8-dodecenyl acetate to 27% decreased the activity further to about 10%. (*Z*)-8-Dodecenyl acetate also decreased the number of successful flights in the flight tunnel.

**Key Words**—*Agrotis segetum*, turnip moth, Lepidoptera, Noctuidae, sex pheromone, flight tunnel, decyl acetate, (*Z*)-5-decenyl acetate, (*Z*)-7-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate, (*Z*)-8-dodecenyl acetate, field tests, attraction.

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## INTRODUCTION

Bestmann et al. (1978) reported (Z)-5-decenyl acetate (Z5-10:OAc) to be a sexual attractant of the turnip moth, *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae), based on chemical analysis, electroantennographic (EAG) activity, and preliminary olfactometer tests. Later, efforts by several groups (Arn et al., 1980, 1983; Toth et al., 1980; Löfstedt et al., 1982) revealed a much more complex, multicomponent pheromone in the turnip moth. Löfstedt et al (1982) identified 13 aliphatic acetates and alcohols from female gland extracts. The pheromone consists of at least the three homologous monounsaturated acetates; Z5-10:OAc, (Z)-7-dodecenyl acetate (Z7-12:OAc), and (Z)-9-tetradecenyl acetate (Z9-14:OAc). In field-trapping experiments these three compounds synergized one another, with no or only slight attractivity as single components or binary mixtures (Arn et al., 1983). Tube olfactometer experiments indicated the possible activity of decyl acetate (10:OAc) (Löfstedt et al., 1982), also present in the female pheromone gland.

Arn et al. (1980) also reported (Z)-8-dodecenyl acetate (Z8-12:OAc) as a predominant constituent of female *A. segetum* pheromone gland extracts, but assigned it an "inhibitory" function. When male *A. segetum* sensilla trichodea were screened (Löfstedt et al., 1982) a receptor cell specialized to Z8-12:OAc and (Z)-5-decenol (Z5-10:OH) was found. We hypothesized that both Z8-12:OAc and Z5-10:OH might have an "inhibitory" effect on *A. segetum* males by activation of a common receptor.

The present study comprises experiments, in a flight tunnel and in the field, designed to elucidate the behavioral activity of the above-mentioned pheromone components and inhibitors.

## METHODS AND MATERIALS

*Insects.* Turnip moths were maintained in the laboratory on a modified Hinks and Byers (1976) diet, using potatoes instead of pea beans. The sexes were separated as pupae, and the males were kept in a separate room at 22°C, 40% relative humidity on a reversed 16-hr light-8-hr dark photoperiod. Each male was kept in a 250-ml plastic jar and fed a 5% sucrose solution. The males tested in the flight tunnel were the 10th- to 15th-generation offspring of insects collected in the field (Löfstedt et al., 1985).

*Chemicals.* Unsaturated compounds used for the formulation of the lures were at least 98% pure regarding geometrical and positional isomers. 10:OAc was purchased from Schuchardt, München, FRG, and (Z)-6-dodecenyl acetate (Z6-12:OAc), Z7-12:OAc, Z8-12:OAc, Z9-14:OAc, and Z5-10:OH from the Institute for Pesticide research, Wageningen, The Netherlands. Z5-10:OAc was a gift from Bernt Thelin, Department of Organic Chemistry 3, Lund Uni-

versity. The rest of the compounds were from the laboratory collection of pheromone components from various sources.

Stock solutions of the single compounds were made up in heptane (1–10 $\mu g/\mu l$). Blends of components to be tested were made by mixing various amounts of the stock solutions and adding hexane to the desired concentration. The mixtures in 25–50 $\mu l$ solvent were applied to rubber septa (red $5 \times 9$ mm, Arthur H. Thomas, Philadelphia, Pennsylvania) or polyethylene capsules (1 ml, 1.5-mm wall thickness, Kartell, Italy). The solvent was evaporated at room temperature, the capsules were closed, and stored at $-20°C$ when not in use. Dosages are designated on their content of *Z*5–10:OAc. For the flight-tunnel experiments with different ratios between the monoenes, fresh baits were made up daily, 4–5 hr before the experiments.

The blend ratio in the 1982 experiments (10:OAc–*Z*5–10:OAc–*Z*7–12:OAc–*Z*9–14:OAc = 1.25:1:12:10) was based on the amounts isolated from female pheromone glands reported in Löfstedt et al. (1982). Later, more precise quantitative analyses of glands revealed a higher proportion of the volatile components (Löfstedt and Odham, 1984 (i.e., 10:OAc–*Z*5–10:OAc–*Z*7–12:OAc–*Z*9–14:OAc = 0.6:1:5:2.5). All experiments during 1983 were based on these new ratios.

*Flight Tunnel Experiments.* Experiments were performed in a 2.0-m-long × 0.9-m-diameter Plexiglas flight tunnel that had a closed design, with the entire air volume exhausted after a single passage through the wind tunnel. Turbulence in the flow was reduced by stainless-steel screens at the outlet as well as at the inlet end of the tunnel. A minor distortion of the flow was observed when the doors at the side of the wind tunnel were opened to introduce baits or moths. The experiment with different ratios between the homologous acetates was performed in an open wind tunnel, with only the contaminated air ventilated out of the room. This tunnel had a square cross-section $0.9 \times 0.9$ $m^2$. Both tunnels had a black and white striped floor.

Flight-tunnel conditions were 20–21°C, 35–40% relative humidity, and 0.25 m/sec wind speed. The light intensity was approximately 1 lux. In the majority of the experiments rubber septa to be tested in the flight tunnel were fixed at one end of a pipe cleaner, ca. 15 cm long, which was attached to the screen at the upwind end of the tunnel. In this way the septum hung about 30 cm above the floor and at least 10 cm from the upwind end of the flight tunnel. Female glands were tested by placing 10 snipped glands on the head of an insect pin and securing the pin to the end of a pipe cleaner. The glands were prepared just prior to testing them in the tunnel. In the experiment with different ratios the septa were placed in a holder at the top of a 35-cm-high steel rod. Two- to 3-day-old male moths to be tested were transferred individually to 250-ml clear plastic cups and placed in the flight tunnel room prior to initiation of the dark period. In this way moths were allowed to acclimate to the low light intensity in the tunnel for at least 3 hr before they were tested 3–5 hr into the scotophase.

For the test a male moth was transferred to a cylindrical screen cage open at one end. Release cages were placed into the tunnel with the open end facing upwind, on a wire screen platform approximately 25 cm above the tunnel floor, 1.5 m from the source. Males were allowed 2 min to respond and were scored for the following behaviors: taking flight, stationary orientation, flight near the release cage, upwind anemotactic flight, and source contact (see Linn and Roelofs, 1981). Behaviors were recorded on a cassette tape recorder.

*Field-Trapping Experiments.* Field tests were conducted in agricultural crops (carrots, lettuce, potatoes, beet roots, or sugar-beets) outside Lund, in southern Sweden.

The 1982 experiments were carried out between June 6 and June 24, using sticky wing traps (Albany Inc., Needham Heights, Massachusetts). In 1983, the field experiments lasted from June 30 to July 12, and a trap similar to the Albany trap but constructed in our laboratory (Löfqvist and Jönsson, in preparation) was used.

Traps within a replicate were spaced at least 20 m apart in a line at a right angle to the predominant wind direction. Replicates were separated by at least 150 m, and usually as much as 300–400 m.

Traps within a replicate were rerandomized as soon as the most attractive trap had caught 5–10 males. In this way positional effects were minimized. The total catch in each trap over the whole experimental period was generally treated as one replicate. Occasionally, replicates in time were generated by dividing a trapping period into two. Trapped moths were counted and removed daily or every second day. The trap bottoms were replaced when they were about to lose their stickiness. The baits were renewed at least every week because of the short half-life of the 10-carbon compounds.

## RESULTS

*Dose–Response Experiments.* In 1982 a blend of 10:OAc, Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc was tested in the field at dosages ranging from 0.001 to 10 $\mu$g (Figure 1). With both polyethylene caps and rubber septa, the highest response occurred with the 10-$\mu$g treatment. The 1983 experiment, using rubber septa, revealed a maximum trap catch over the range of 0.3–30 $\mu$g. (Figure 1).

The 1983 ratio was also tested in the flight tunnel. Males responded in highest numbers to 3- and 30-$\mu$g treatments, with 54 and 59%, respectively, of the males flying upwind and making contact with the source (Figure 2). This response level was significantly lower, however, than that observed to female glands. Increasing the dose to 300 $\mu$g caused arrestment of upwind flight as well as a significant decrease in the number of males that initiated upwind movement, while decreasing the dose lowered the number of males orienting in the odor

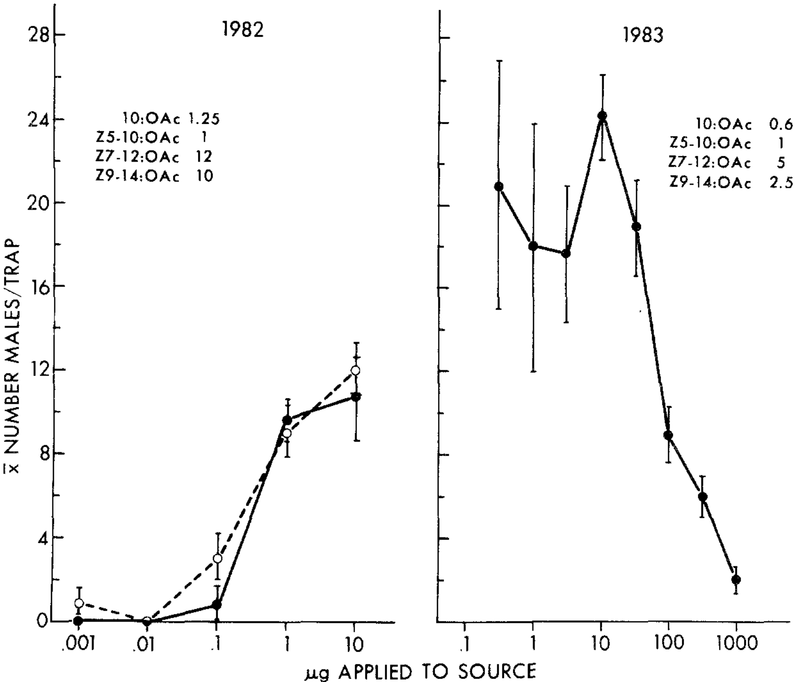



FIG. 1. Mean number of *A. segetum* males (± SEM) attracted to different dosages of two mixtures of 10:OAc, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc ($N = 4$ in both experiments). For 1982 the dashed line is for polyethylene caps, while the solid line for 1982 and 1983 is for rubber septa.

plume, with all of the males that successfully oriented also completing the upwind flight. Temporal analysis of the behavioral response showed that the time to proceed from the initial behavior, activation (wing fanning), to any succeeding step in the flight sequence increased with increasing amounts of stimulus (Figure 3), with the shortest duration for the behavioral sequence observed to female glands.

*Tests with Additional Components.* Several additional blends were tested during the 1982 field season (Figure 4). The mixtures containing the unsaturated acetates Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in a 1:1:1 ratio were most attractive (blends 1 and 2). As in the subtractive tests, leaving out 10:OAc did not affect trap catches (blends 1 and 3 compared to blends 2 and 4, respectively), while the omission of Z9-14:OAc did (blend 5 compared to blend 4). The relative activity appeared to be restored by the addition of a large amount of Z9-12:OAc (blends 6 and 7 relative to blend 4). The addition of Z5-10:OH (blend 8) or Z8-12:OAc (blend 9) reduced the trap catches. In 1983 a separate field

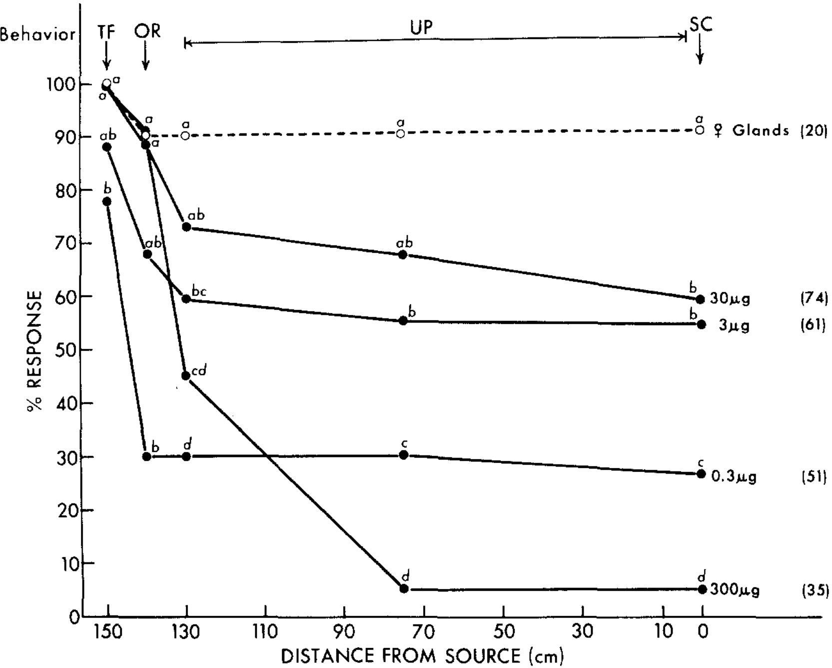



FIG. 2. Percentage response of male *A. segetum* tested individually in the flight tunnel to female glands and four dosages of a synthetic four-component mixture of 10:OAc, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc (0.6:1:5:2.5). The dose (μg) refers to the amount of Z5-10:OAc in the respective source. Behaviors are: taking flight (TF), orientation flight (OR), upwind flight (UP), and source contact (SC). Values in parentheses indicate the number of males tested to each treatment. Data points within each behavior having different letters are significantly different, according to the method of adjusted significance levels for proportions (Ryan, 1960). ($P < 0.05$).

experiment was designed to study the effect of the potential "inhibitor" Z8-12:OAc and a neutral compound, the isomer Z6-12:OAc, on trap catches. Whereas even small amounts of Z8-12:OAc reduced the trap catches, no such effect was observed for Z6-12:OAc (Figure 5). When 150 μg of Z8-12:OAc (= the amount of the pheromone component Z7-12:OAc) was added to the four-component mix at the 30-μg dose, the percentage of males taking flight decreased from 96 to 75%. The proportion of males reaching the source decreased from 60 to 0% ($N = 25$).

*Subtractive Assay.* Removing any of the three unsaturated acetates from the 3-μg dosage of the four-component blend tested in the 1983 field trials resulted in significantly fewer males being trapped (Figure 6). In contrast, the omission of 10:OAc from the blend did not significantly change the trap catch.

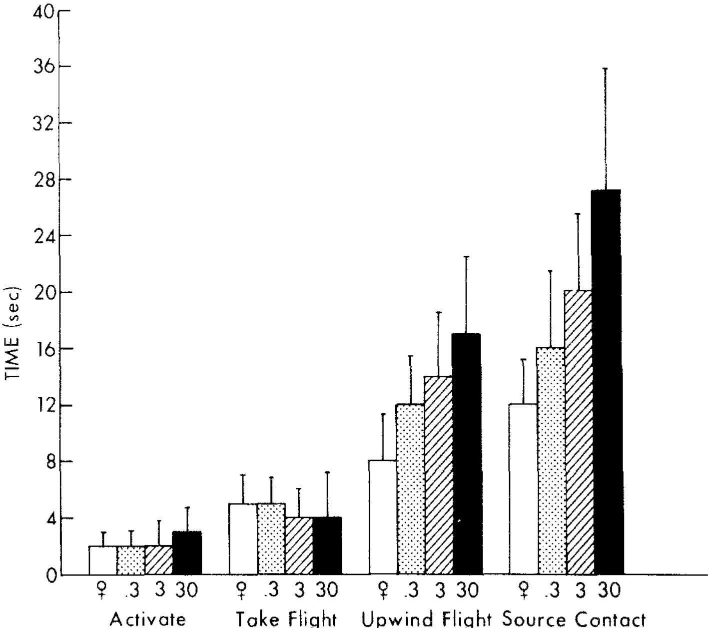


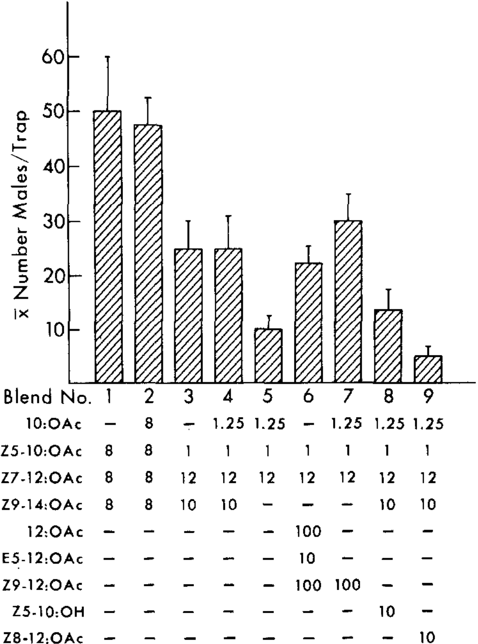

FIG. 3. Time (seconds) taken by male *A. segetum* to initiate four phases of the behavioral response to treatments in Figure 2 (300-μg dose excluded). Values are the means (± SEM) for the number of males completing the sequence to each treatment (see Figure 2).

In flight-tunnel tests with a 30-μg dosage of the 1983 four-component blend, a pattern similar to that in the field was observed (Figure 7). However, in the flight tunnel removal of Z9-14:OAc (treatment 5) appeared to have a more dramatic effect on the behavior than was observed in the field.

*Comparison of Two Ratios of Homologous Acetates.* We compared the response of males to the 1:5:2.5 blend used by us with the response to the 1:1:1 blend found to be optimal by Arn et al. (1983). At three different dosages (Table 1) there were no significant differences between the two blends. The maximal response was in both cases obtained with the 3-μg dosage. At the 30-μg dosage many of the males initially orienting into the plume never completed the upwind flight. Temporal analysis of the responses was not performed.

## DISCUSSION

The optimal pheromone dose for attraction of *A. segetum* males in our experiments was within the 1- to 30-μg range, both in the field and in the flight




| Blend No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| 10:OAc | – | 8 | – | 1.25 | 1.25 | – | 1.25 | 1.25 | 1.25 |
| Z5-10:OAc | 8 | 8 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Z7-12:OAc | 8 | 8 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Z9-14:OAc | 8 | 8 | 10 | 10 | – | – | – | 10 | 10 |
| 12:OAc | – | – | – | – | – | 100 | – | – | – |
| E5-12:OAc | – | – | – | – | – | 10 | – | – | – |
| Z9-12:OAc | – | – | – | – | – | 100 | 100 | – | – |
| Z5-10:OH | – | – | – | – | – | – | – | 10 | – |
| Z8-12:OAc | – | – | – | – | – | – | – | – | 10 |

FIG. 4. Mean number of *A. segetum* males (± SEM) attracted to blends of pheromone candidates ($N = 4$). Values indicate the amount ($\mu$g) of each compound applied to rubber septum source.

tunnel. Field results using rubber septum and polyethylene capsule dispensers gave similar results, although half-lives of pheromone components on rubber septa (Butler and McDonough, 1979) are much longer than on polyethylene capsules (Kuhr et al., 1972). In addition, changes in component ratios between 1982 and 1983 did not seem to affect the optimal dose substantially. In both years maximum trap catch was achieved with dosages that correspond to 200–300 $\mu$g total dispenser load. Arn et al. (1983) found maximum attraction to a 1 : 1 : 1 mixture of the monounsaturated acetates (10 : OAc not included) with a 300-$\mu$g total septum load. Thus the optimal dose seems to be relatively independent of the blend ratios.

The flight-tunnel experiments confirmed that, as in earlier studies on the Oriental fruit moth *Grapholita molesta* (Baker and Roelofs, 1981; Linn and Roelofs, 1983), lower and upper behavioral thresholds (Roelofs, 1978) are due to decreased activation and arrestment of upwind flight, respectively. Temporal analyses of the male response added an important measure to the behavioral analysis. The increase in time necessary to proceed through the steps in the behavioral chain corresponds with the decreased ground velocity as an effect of increased pheromone concentration reported for flying moths (Cardé and Hagaman, 1979; Kuenen and Baker, 1982).

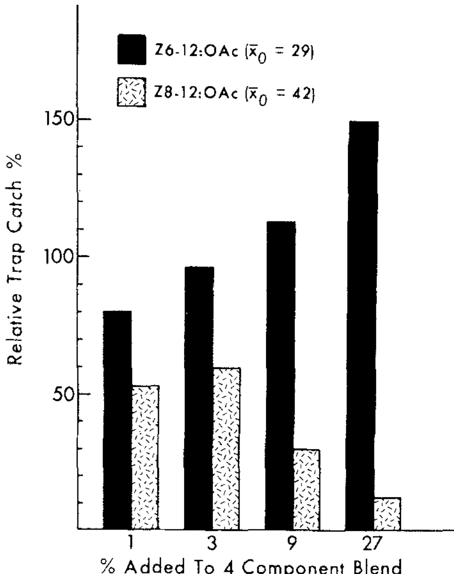



FIG. 5. Mean trap catch ($N = 4$) relative to the four-component blend (10:OAc, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc; 0.6:1:5:2.5) with two pheromone component analogs added. $\bar{X}_0$ = mean trap catch with four-component control in the experiment with the respective analog. The experiments were performed with the 3-$\mu$g dosage of the pheromone. Percent added is related to the total amount of the four-component blend, i.e., 27 $\mu$g.

From the present study, it is not possible to assign a specific function to any of the *A. segetum* pheromone components. Subtraction of any of the three monounsaturated acetates affected all behavioral steps from activation to contact with the odor source, while 10:OAc seemed to be behaviorally neutral. Thus our study adds evidence to a more holistic perspective, with the total signal emitted affecting all steps in the behavioral response (Linn and Roelofs, 1983; Linn et al., 1984). Flight-tunnel and field data also correspond in this respect, except for the subtraction of Z9-14:OAc. Here the trap catch is higher than would be expected from the flight-tunnel data. However, the approximately 50% reduction in trap catch is consistent with what Arn et al. (1983) found in their field test with the three unsaturated homologs in Denmark, close to southern Sweden. It is interesting that Z9-12:OAc seemed to substitute for Z9-14:OAc in the blend, when added in ten times higher amounts. This could be because of interaction with the Z9-14:OAc receptor (Löfstedt et al., 1982).

Even our most active synthetic blend tested in the flight tunnel was inferior to pinned female glands. Studies with *Trichoplusia ni* (Linn et al., 1984) have shown that it should be possible to get a near 100% response with synthetic compounds in an assay of this kind. It should also be pointed out that the males responded faster to the glands than to any of the four-component dosages and

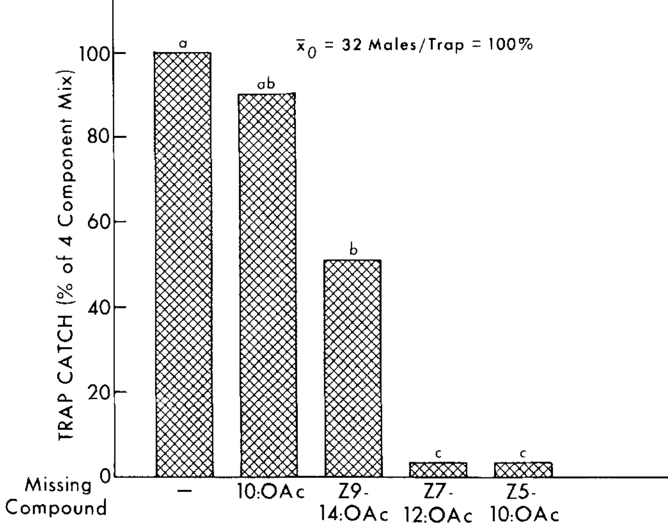



FIG. 6. Mean trap catch ($N$ = 4) relative to a four-component blend of 10:OAc, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc (0.6:1:5:2.5) with one of the compounds subtracted. Significant differences are based on ANOVA ($\sqrt{X + 0.5}$ transformations of the total catch) followed by comparison of means by Duncan's multiple-range test. $\bar{X}_0$ = average catch with four component blend.

that the longest duration for the complete behavioral sequence was observed to the peak 30-$\mu$g synthetic blends. This can be compared to observations on *G. molesta*, in which the duration of each phase of the behavioral sequence increased with the deviation from the optimal blend (Linn and Roelofs, 1983). We think that an optimal stimulus is characterized not only by high numbers of responding individuals, but also by a rapid response.

In an attempt to rectify our use of what could have been a suboptimal ratio between the monoenes, the 1:5:2.5 blend was compared with the 1:1:1 blend found to be optimal by Arn et al. (1983). A full exploration of different ratios and the possible impact of the pheromone dose on the optimal ratio (Linn and Roelofs, 1983; Bellas and Bartell, 1983) was not within the scope of this study. The experiment reported did not reveal any dramatic effect of the ratios on the number of males responding. None of the blends elicited completed upwind flight in more than 50% of the males. In this experiment males responding to the 30-$\mu$g dose of the 1:5:2.5 blend did not fly upwind to the same extent as in the first dose–response experiment. A probable explanation for this is the use of fresh baits every day, resulting in relatively higher release rates.

We can see several explanations for the lower activity of synthetics in our assay. First, although the present study and other reports (Arn et al., 1983; Löfstedt et al. 1985) have shown that the ratios between components is of minor

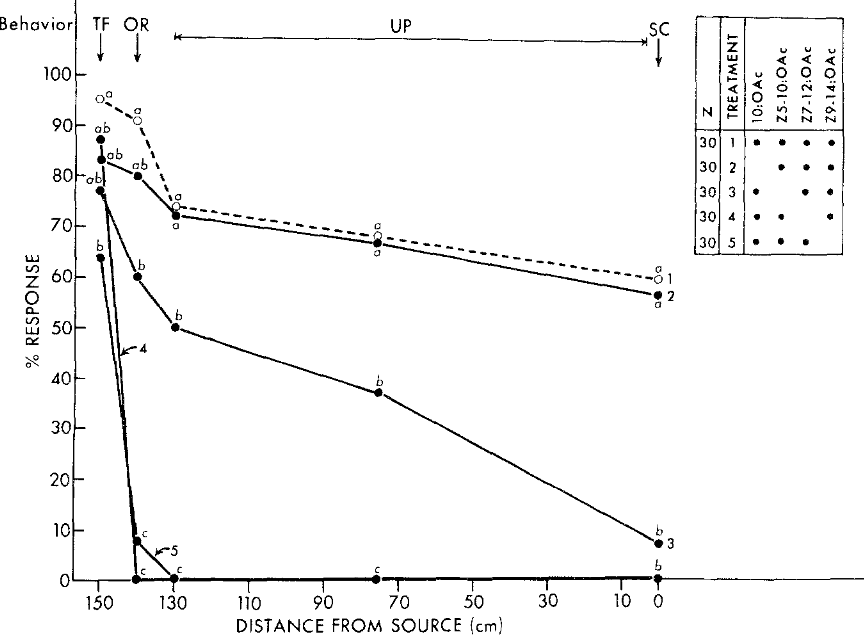



FIG. 7. Percentage response of male *A. segetum* tested individually in the flight tunnel to 30-$\mu$g dosage of the four-component blend and the four three-component blends in Figure 6. Behaviors and analysis as in Figure 2.

TABLE 1. RESPONSES OF MALE *Agrotis segetum* IN FLIGHT TUNNEL TO TWO BLEND RATIOS OF Z5-10:OAC-Z7-12:OAC-Z9-14:OAC AT DIFFERENT DOSAGES

| Dose ($\mu$g) | Ratio (Z5-10:OAc-Z7-12:OAc-Z9-14:OAc) | Response (%)[a] Number tested | Taking flight | Stationary orientation | Source contact |
|---|---|---|---|---|---|
| 0.3 | 1:5:2.5 | 50 | 100 | 70a | 34ab |
| | 1:1:1 | 50 | 100 | 62a | 30ab |
| 3 | 1:5:2.5 | 53 | 100 | 64a | 34ab |
| | 1:1:1 | 73 | 100 | 75a | 51a |
| 30 | 1:5:2.5 | 50 | 100 | 64a | 16b |
| | 1:1:1 | 55 | 100 | 56a | 24b |

[a]Values in each column followed by the same letter are not significantly different ($P < 0.05$) according to the method of adjusted significance levels for proportions (Ryan, 1960).

importance, the discord in release rates from glands and rubber septa might be enough to explain the difference. This can be tested by collection of airborne volatiles from dispensers and glands.

Second, pheromone components might be missing in the blend. Addition of Z9–12:OAc, 12:OAc, and Z5–10:OH did not increase the activity significantly, but more aliphatic acetates and alcohols with possible pheromone activity are present in the gland (Löfstedt et al., 1982). Furthermore, it is not unlikely that trace components, in addition to these reported, are present. Synergistic affects of less than 0.1% have been shown in Lepidoptera (Steck et al., 1982). Unfortunately the very low amounts of pheromone per female ($\leq 3$ ng) makes analytical work with *A. segetum* demanding. Finally, it cannot be ruled out that impurities in the compounds ($<2\%$) used to make up the synthetic blends could account for the reduced activity.

"Inhibition" of pheromone behavior is a poorly understood area of research. Nonpheromone chemicals that negatively influence attraction have been referred to as "inhibitors," "maskers," or "antiattractants" in various contexts (Cardé, 1976). Our study reveals the mode of action of Z8–12:OAc on male *A. segetum* pheromone behavior as mainly arrestment of upwind flight. In contrast the Z6–12:OAc isomer did not have this negative effect but rather increased the attraction, while Z5–10:OH, which activates the same receptor as Z8–12:OAc, also reduced trap catch. Obviously a negative activity cannot be assigned to every pheromone analog. Arrestment of upwind flight evoked by activation of a specific receptor cell is probably too simple an explanation, but it remains a testable and nonfalsified working hypothesis.

We can imagine at least two groups of negative compounds: ecologically meaningful compounds, important in intra- and/or interspecific communication, and synthetic compounds not naturally occurring. The latter group is of no ecological relevance but might still be of potential use in pest control. Z8–12:OAc is not a very common pheromone component in moths; it has only been found in a few tortricids (Roelofs and Brown, 1982), and the adaptive significance of a male receptor cell tuned to such a compound that is not used in the sex pheromone is unclear.

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# NOVEL COMPONENTS FROM SECRETORY HAIRS OF AZALEA LACE BUG *Stephanitis pyrioides* (HEMIPTERA: TINGIDAE)

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**Abstract**—The azalea lace bug secretes a clear fluid from secretory setae on the antennae and globulated spines on the dorsal and lateral aspects of the abdomen. The secretion contains 2-alkyl-5-hydroxychromones, the corresponding chromanones and diketones, and straight-chain aldehydes and ketones.

**Key Words**—Heteroptera, Tingidae, *Stephanitis*, lace bug, setal exudate, chromones, chromanones.

## INTRODUCTION

Nymphs and adults of the lace bug *Stephanitis pyrioides* (Scott) feed on the undersides of azalea leaves. In spite of the gregarious feeding and social habits of lace bugs (Drake and Ruhoff, 1965), neither parasites nor predators of nymphs of *S. pyrioides* have been reported. There are incidental reports of predation of *Stephanitis* species by an occasional hemipteran (e.g., Johnson, 1936), but Sheeley and Yonke (1977) failed to find any significant numbers of either parasites or predators for seven species of Missouri tingids. Although defensive substances associated with lace bugs have not been reported, it is known (Livingstone, 1978) that nymphs secrete a clear, slightly viscous fluid from setae or hairs that are generally distributed laterally and dorsally on abdominal protruberances as well as on segments of the antennae. These microdroplets are supported and remain in place on the end of the hairs (Figure 1). We here report the identification and synthesis of the major chemical constituents of secretions of nymphs of *S. pyrioides*.



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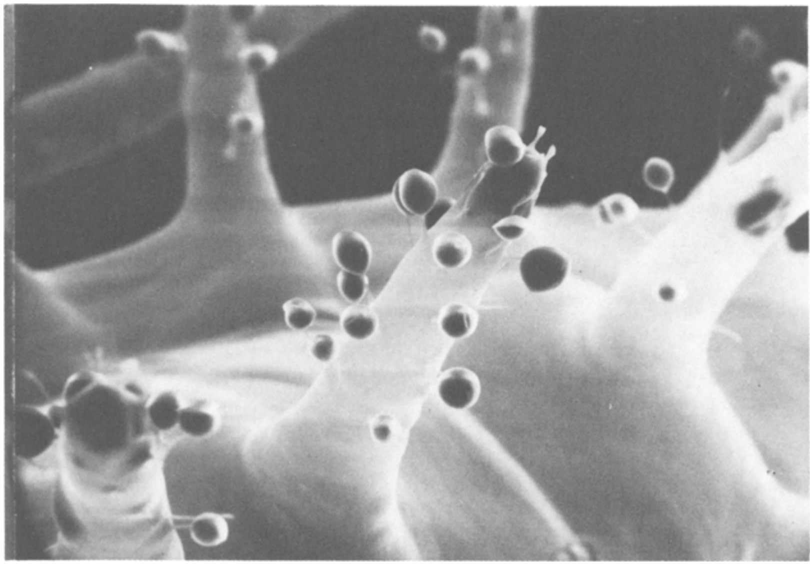

FIG. 1. Exuviae of *S. pyrioides* fourth instar with tubercles (scoli) with globulated spines.

## METHODS AND MATERIALS

Gas chromatography was performed on a Varian model 3700 instrument equipped with a flame ionization detector and a 13 m capillary DB-1 column (J & W Scientific, Inc.) with helium as carrier. The injector temperature was 270°C and the column was heated either isothermally or programed over a range of 100–245°. Mass spectra were obtained from a Finnigan model 4510 GC-MS-DS fitted with a 30 mm × 0.32 mm ID DB-1 (0.25 $\mu$m film of methyl silicone) fused silica column. Spectra were collected at 70 eV and a source temperature of 150°. High-performance liquid chromatography was performed on a Spectra-Physics model 8700 system equipped with a 30 cm × 3.9 mm $C_{18}$ $\mu$-Bondapak column eluted with 85% methanol containing 0.005 M tetrabutylammonium phosphate.

A colony of *Stephanitis pyrioides* (Scott) was maintained in a greenhouse on container-grown azaleas (Krume hybrid, "Blaauw's Pink"); third through fifth instar nymphs were used for collections. Microdroplets were blotted from the dorsal abdominal aspects of laboratory-reared nymphs with small strips of filter paper, and dichloromethane extracts of the filter paper were examined by capillary gas chromatography, gas chromatography–mass spectrometry, and high-performance liquid chromatography.

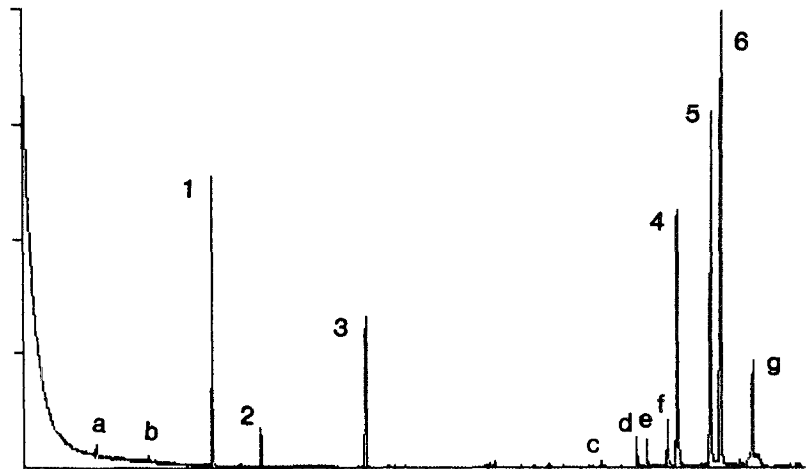


FIG. 2. Reconstructed ion chromatogram of compounds secreted by *S. pyrioides*. The components assigned numbers were positively identified and are described in the text and in Scheme 1. Structures of the components assigned lowercase letters have not been confirmed but had been tentatively proposed to be: a, octanal; b, nonan-2-one; c, 2-heptyl-5-hydroxychroman-4-one; d, 2-heptyl-5-hydroxychromone; e, 2′,6′-dihydroxy-2-octanoylacetophenone; f, unidentified; g, dioctyl phthalate.

## RESULTS

A reconstructed ion chromatogram of the nymph exudate is shown in Figure 2. Decanal (1) and undecan-2-one (2) were initially identified by computer-selected matches of their mass spectra. The data system suggested 2′,4′-dihydroxyacetophenone as a likely structure for the next component (*m/z* 152, 137). However, comparison with authentic (the 2′,4′-, 2′,5′-, and 2′,6′-isomers were available from the Aldrich Chemical Company), showed that the secreted compound was in fact the 2′,6′-isomer (3). The three later-eluting major components 4–6 were unknown, but their mass spectra indicated molecular weights of 290, 288, and 306, respectively (molecular weights of 4–6 were confirmed by chemical ionization mass spectrometry using both methane and ammonia as reagent gases); furthermore, an intense ion at *m/z* 137 (dihydroxybenzoyl?) in the electron impact mass spectrum of each suggested that all three were related to or derived from 3.

Both chromones (Eguchi, 1979; Ellis, 1977a) and chromanones (Van de Sande and Vandewalle, 1973; Ellis, 1977b) are known to undergo facile reverse Diels-Alder fragmentations upon electron impact; thus a 2-alkyl-5-hydroxy derivative of either ring system might be expected to produce a mass spectral

3 R = R' = H
6 R = H, R' = $COC_9H_{19}$
7 R = $CH_2Ph$, R' = H
8 R = $CH_2Ph$, R' = $COC_9H_{19}$

3 —a→ 7 —b→ 8 —c→ 6 —d→ 5 —c→ 4

a. $PhCH_2Cl$, $K_2CO_3$, b. $C_9H_{19}CO_2Et$, NaH, Py. c. $H_2$ Pd/C, d. HCl

SCHEME 1. Structures and synthesis of identified compounds.

fragment of $m/z$ 137 equivalent to that produced by 3. A reported (Tringali and Piattelli, 1982) mass spectrum of 5,7-dihydroxy-2-nonadecylchromone contains a series of homologous ions completely parallel to those found in the mass spectrum of 5, differing by 16 amu because of one less oxygen on the ring of 5 [principal ions of 5 occurred at $m/z$ 288 (35%), 189 (100%), 176 (57%), 147 (10%), 137 (78%), 108 (12%)]. None of the alkyl ions produced by 5 suggested branching, and we proposed 5-hydroxy-2-nonylchromone as its structure (Scheme 1).

Compound 4 proved to be 2 amu heavier than 5. The electron impact mass spectrum of 4 contained a molecular ion at $m/z$ 290 (44%) and fragments at 163 (93%), 137 (100%), 108 (12%), and we proposed the chromanone structure illustrated in Scheme 1.

The mass spectrum of the major component 6 had an ion (relative intensity 100%) at $m/z$ 179, a moderately itense ion at $m/z$ 137 (63%), and relatively weak ions at $m/z$ 306 (molecular ion, 5%), 189 (9%), 152 (13%), and 108 (10%). Briefly warming a sample of the mixture with HCl resulted in conversion of 6 to 5, consistent with the known facile cyclization of such diketones to chromones (Ellis, 1977c).

Assignments of structures 1–3 were confirmed by comparing gas chromatographic retention times and mass spectra to those of commercial samples. Compounds 4–6 were synthesized as outlined in Scheme 1: monobenzyl ether 7 (Kametani and Kano, 1963) and ethyl decanoate were condensed with sodium hydride in pyridine (Cooke and Down, 1971) to give 8 (81%, mp 50.5-52.5°). Hydrogenolysis of 8 (Pd on carbon 1 atm in ethanol containing a little triethylamine) gave 6 as a very pale yellow solid, mp 71.5–73.5° after recrystallization from hexane plus a small amount of benzene [$^1$H]NMR ($CDCl_3$) $\delta$ 0.90 (t,3H), 1.31 (m,14H), 2.10 (m,2H), 2.88 (s,2H), 6.23–7.41 (m,3H), 11.61 (s,2H). A sample of 6 (0.28 g) was boiled 3–5 min in a mixture of conc. HCl (2 ml) and ethanol (4 ml); after cooling to 0°, the solid was collected and recrystallized

from wet methanol to give 0.21 g of 5, mp 51–52°. [$^1$H]NMR ($CDCl_3$) $\delta$ 0.88 (t,3H), 1.29 (m,14H), 2.55 (m,2H), 6.09 (s,1H), 6.63–7.63 (m,3H), 12.45 (s,1H).

As has been discussed by Ellis (1977d) and others, hydrogenation of the 2,3 double bond of chromones is slow, and competitive reduction of the carbonyl often occurs. To obtain chromanone 4, hydrogenation of 5 (Pd.C, Et0Ac-Et0H, 1 atm) was followed by periodic gas chromatographic analyses until the relative concentration of 4 no longer increased. Silica gel chromatography (hexane with increasing increments of benzene) then gave a pure sample of *dl*-4 (eluted with 60–80% benzene) that was an oil room temperature but formed a white solid at 0°. [$^1$H]NMR ($CDCl_3$) $\delta$ 0.89 (t,3H), 0 (m,16H), 2.69 (d, $J$ = 7 Hz, 2H), 4.3 (m,1H), 6.3–7.4 (m,3H), 11.65 (1H). Gas chromatographic retention times and mass spectra of synthetic 4–6 perfectly matched those of the insect-derived materials. In addition, 5 and 6 were further confirmed by RP-HPLC; 4 was not detected in this particular sample.

A somewhat less laborious and more quantitative method of collecting the material was to simply dip the nymphs and/or their cast moult skins in a vial containing dichloromethane. However, such solutions were usually contaminated with varying amounts of additional components including saturated hydrocarbons that may have been of insect cuticule origin. From 25 cast skins from fifth-instar nymphs, we extracted an estimated (by GLC) total of 18 $\mu$g 5 + 6, i.e., ca. 0.8 $\mu$g/nymph. The average live weight of fifth-instar nymphs was determined to be 0.38 mg; thus 5 and 6 seem to constitute approximately 0.2% of their body weight.

Compounds 1, 4, 5, and 6 were readily discernible by GLC in nearly all samples examined, whereas the concentrations of 2 and 3 seemed somewhat more variable. Some cyclization of 6 to 5 was found to occur during GLC analyses; this was not a problem during HPLC analyses, however, and the latter demonstrated that both compounds were present in all insect-derived samples examined. The minor components indicated by lowercase letters in Figure 2 were lost in the baseline noise of many of the samples, and these compounds have not been investigated in detail. Their mass spectra are strongly suggestive of a homologous series of compounds with the alkyl groups shorter by two carbons.

Compounds 4–6 have not previously appeared in the literature. We considered the possibility that the insects were obtaining them from the azalea leaves, but we have no evidence that this was the case. Extraction of freeze-dried azalea leaves followed by column chromatography and GLC analysis of fractions with TLC $R_f$s corresponding to those of 4–6 indicated that measurable amounts of 4–6 were not present in the host plant. However, we have not ruled out the possibility that they might have been present as conjugates. In one case, we examined the secretion of a group of nymphs of *S. pyrioides* found locally on an ornamental rhododendron (*Rhododendron catawbiense* "Boursault," i.e., a

different species of host plant). The composition of this secretion was indistinguishable from that derived from the nymphs feeding on azaleas.

We believe that this constitutes the first identification of tingid exocrine compounds and the first report of either chromones or chromanones from insects. Studies on the function of these compounds are in progress as are investigations of secretions of additional species of lace bugs. Our preliminary results suggest that the rhododendron lace bug, *Stephanitis rhododendri* (Horvath), produces a mixture that contains diketone 6 and several related compounds. In contrast, those representatives of the *Corythucha* genus thus far examined [*C. cydoiae* Fitch (Hawthorn lace bug), *C. arcuata* Say (Oak lace bug), and *C. ulmi* Osborne and Drake (Elm lace bug)] have been found to produce a series of as yet unidentified compounds related to each other but apparently unrelated to those found in the *Stephanitis* genus.

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# IDENTIFICATION OF FEEDING STIMULANTS FOR BOLL WEEVILS FROM COTTON BUDS AND ANTHERS[1]

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**Abstract**—Column chromatography of the pentane extract of freeze-dried cotton buds or anthers yielded a wax–sterol ester fraction that exhibited potent feeding stimulant activity for the cotton boll weevil. The waxes of the wax–sterol ester mixture were responsible for the feeding activity. Saponification of the wax–sterol ester fraction yielded about 15% alcohols and 85% sterols. A $C_{18:1}$ alcohol, dihydrophytol, phytol, and geranylgeraniol constituted 15, 36, 26, and 23%, respectively, of the total alcohols, implicating certain of their long-chain esters as feeding stimulants. Several esters of dihydrophytol, phytol, and geranylgeraniol were identified among the waxes by GC-MS. Certain phytol, geranylgeraniol, and oleyl alcohol esters containing $C_{12}$ to $C_{26}$ acid moieties were synthesized and were found to induce high feeding stimulant activity in the cotton boll weevil.

**Key Words**—Boll weevil, *Anthonomus grandis*, Coleoptera, Curculionidae, feeding stimulants, cotton buds, anthers, phytol, geranylgeraniol esters, phytol oleate, phytol dodecanoate.

## INTRODUCTION

Since Keller et al. (1962) reported the presence of feeding stimulant(s) for boll weevils, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), in water extracts of cotton squares, considerable work has been done toward the identi-

[1]In cooperation with the Mississippi Agricultural and Forestry Experiment Station.



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fication of compounds that stimulate feeding activity. Hedin et al. (1974) furnished an excellent review of the work on insect feeding stimulants in general, including compounds that stimulated feeding activity of the boll weevil. Several workers reported on a number of compounds from the cotton plant that elicited some degree of feeding in the boll weevil (Hedin et al., 1966; Stuck et al., 1968a, b; Temple et al., 1968). None of the studies, however, have identified compounds of sufficient activity to be considered as the feeding stimulant for the boll weevil. This paper reports on the identification and synthesis of compounds from cotton buds and anthers that are highly active feeding stimulants for the boll weevil.

## METHODS AND MATERIALS

*Extraction of Cotton Buds and Anthers.* Whole cotton buds with bracts were chopped in a blender and then freeze-dried. Anthers were dissected out of buds and then freeze-dried whole. The freeze-dried materials were extracted in a Soxhlet apparatus for 3 hr successively with pentane, ethyl acetate, chloroform, and methanol. The solvents were removed under reduced pressure at 50°C, and the resulting residues were bioassayed.

*Feeding-Stimulant Bioassay.* Laboratory reared boll weevils, 1 to 4 weeks old, were used in the bioassays. A specific quantity of residue, equivalent to one bud or androecium (aggregate of anthers), of an extract or chromatographic fraction was dissolved in 1 ml of hexane or methanol. For bioassay of the synthetic esters, a 0.04 M solution in hexane was prepared. Approximately 20 $\mu l$ of each test solution was placed within two of four 1-cm-diameter circles drawn around the periphery of 7-cm-diameter qualitative grade filter paper. Similarly, solvent blank was applied to the two remaining 1-cm circles. After the solvent evaporated, the filter paper was placed inside an 11-cm-diameter Petri dish on a 0.5-cm layer of 2.5% agar. Ten insects were placed into each dish, and the dishes were placed in a dark environment overnight at 29°C. Feeding response was determined by totaling the number of feeding punctures within the treated circles then subtracting the total number of punctures in the blank. Twenty replicas were obtained.

*Chromatography.* The residue from the pentane extractions was chromatographed over 25 g of hexane-washed Merck silica gel (2.5 × 13 cm), and the column was eluted with 100-ml volumes of 0, 3, 10, 25, and 50% ether in hexane. Of the 3% ether in hexane eluent, fractions of 75-ml and 25-ml volume were collected. TLC analyses were conducted on silica gel plates. The solvent systems were toluene–hexane (1 : 1) and toluene–ethyl acetate (9 : 1).

*Feeding Stimulant Analyses by GLC and GC-MS.* Natural and synthesized feeding stimulants were analyzed by GLC and GC-MS. GLC was performed with a Varian model 3700 gas chromatograph equipped with a 15-m × 0.32-

mm DB-1 fused silica capillary column (0.25 $\mu$m film). Mixtures of esters were injected onto the column at 240°C, programed, after an initial hold of 1 min, 10°/min to 285° and held; total time 40 min. For the alcohols, the program was 1 min at 70°C, 10°/min to 285°C and held for 20 min. GC-mass spectral data were obtained with a Finnigan model 4500 spectrometer fitted with a 15-m × 0.25-mm DB-1 fused silica capillary column (0.1 $\mu$m film thickness) and connected to an Incos data system. Mixture of esters or alcohols were injected at 240°C, held for 1 min, then programed at 10°/min to 290°C and held as in the capillary GLC analyses. The compounds were analyzed by ammonia chemical ionization and electron impact mass spectrometry.

*Saponification of Esters.* The esters were saponified by refluxing with 5% potassium hydroxide in methanol–benzene (5 : 1) for 4 hr. Most of the solvent was removed under vacuum and the mixture was diluted with water, acidified with dilute hydrochloric acid, and extracted three times with hexane. The hexane solution was washed with water, dried over sodium sulfate, and the hexane removed under vacuum. The dried residue was dissolved in ether and treated with an excess of diazomethane to react with fatty acids. The ether was removed under vacuum, and the residue was chromatographed.

*Synthetic Esters.* The esters listed in Table 3 were prepared via reaction of the acid with an excess of thionyl chloride in benzene at reflux temperature. After removal of solvent and excess thionyl chloride, the acid chloride was redissolved into dry benzene and added to a gram equivalent of the alcohol and pyridine in dry benzene. The reaction mixture was heated overnight at 60°C, then solvent was removed under vacuum and the dry residue was triturated with hexane. The hexane-soluble material was chromatographed over activity grade II alumina and eluted with hexane and 5%, and 10% ether in hexane. The fractions were analyzed by TLC, and the fractions showing only one component were further analyzed by capillary GLC before the purified ester fractions were combined.

## RESULTS AND DISCUSSION

*Extracts of Buds and Anthers.* When cotton buds or anthers were extracted with pentane, ethyl acetate, chloroform, and methanol, the pentane extracts of either buds or anthers elicited the highest feeding response. The methanol extract of anthers was also active (Table 1). The pentane and methanol extractions of 25 g of freeze dried buds (193) yielded 0.69 g and 4.72 g, respectively, whereas similar extractions of 25 g of freeze-dried anthers (1000 androecia) gave 1.96 g and 6.37 g, respectively. The great differences in the polarities of pentane and methanol indicate that there are structurally different feeding stimulants for the boll weevil in cotton buds and anthers. Our results only deal with those identified from the pentane extracts.

TABLE 1. RESPONSE OF BOLL WEEVILS PRESENTED CRUDE EXTRACTS OF COTTON BUDS AND ANTHERS[a]

| | Number of feeding punctures | |
|---|---|---|
| Extracts | Buds | Anthers |
| Pentane | 92 | 157 |
| Methanol | 17 | 88 |
| Blank | 12 | 3 |

[a] The quantity of mass applied to filter paper from the pentane and methanol extracts from buds were 72 and 489 μg, respectively; from anthers 39 and 127 μg respectively.

*Chromatography of Pentane Extract and Identity of Active Fraction.* When pentane extracts from cotton buds or anthers were chromatographed and the fractions bioassayed, fraction 2, which was eluted with the 75-ml volume of 3% ether in hexane and contained only trace quantity of mass, was inactive; however, the material eluted with the last 25-ml volume of 3% ether in hexane (fraction 3) showed high feeding stimulant activity (Table 2). These fractions contained approximately 20% and 22.5% of the total residue from the pentane extract of cotton buds and anthers, respectively. The materials had TLC $R_f$'s similar to those of authentic waxes and sterol esters. Infrared analyses of the materials also supported the structures of waxes and sterol esters.

*Indirect Identification of Waxes as Feeding Stimulants.* Since it was not possible to physically separate the waxes from sterol esters in sufficient quantities for testing each component for feeding stimulant activity, the wax-sterol ester mixture was saponified. The aliphatic alcohols and sterols were separated into an alcohol-4,4-dimethylsterol fraction and a desmethylsterol fraction. Reconversion of each fraction to a mixture of esters consisting of $C_{14}$ and $C_{16}$ acid

TABLE 2. RESPONSE OF BOLL WEEVILS PRESENTED CHROMATOGRAPHIC FRACTIONS OF PENTANE EXTRACTS OF COTTON ANTHERS[a]

| Fractions | Number of feeding punctures (anthers) |
|---|---|
| 1 | 22 |
| 2 | 20 |
| 3 | 403 |
| 4 | 22 |
| 5 | 24 |
| 6 | 24 |

[a] The quantity of material in fraction 3 applied to the test filter was 9 μg.

moieties (tetradecanoic and hexadecanoic acids were two of the major fatty acids of the saponified wax–sterol ester mixture) followed by feeding stimulant assays showed that the esters of the alcohol-4,4-dimethylsterol fraction were active. A later assay of the $C_{14}$ and $C_{16}$ ester mixture of the purified 4,4-dimethylsterol was found to be inactive, indicating that the waxes of the wax-sterol ester mixture were responsible for the feeding stimulant activity.

*Identities of Alcohol Moieties of Waxes.* Analyses by capillary GLC of the alcohol–sterol mixture obtained from the saponification of the mixtures of waxes and sterol esters from cotton buds or anthers were quite similar and indicated that the aliphatic alcohol and sterol contents represented 15 and 85%, respectively. There were essentially only four aliphatic alcohols, and analysis by CI-MS and EI-MS of the alcohols with retention times of 11.92, 12.15, 12.45, and 13.08 min and representing approximately 15, 36, 26, and 23%, respectively, of the total nonsteroidal alcohols, indicated molecular masses of 268, 298, 296, and 290. They were identified as a $C_{18}$ alcohol with a double bond, dihydrophytol, phytol, and geranylgeraniol, respectively. Authentic (Z)-9-oleyl alcohol, dihydrophytol, phytol, and geranylgeraniol had retention times of 11.96, 12.22, 12.5, and 13.13 min, respectively. The electron impact and ammonia chemical ionization mass spectra of the authentic alcohols and alcohols from cotton buds or anthers were identical. The retention times of sterols were from 19 to 23 min.

*Analyses of Waxes of Wax–Sterol Ester Fraction by GLC and GC-MS.* The active wax–sterol ester fraction from cotton buds or anthers, when analyzed by capillary GLC, gave similar chromatograms, although the chromatogram of anthers exhibited fewer minor peaks. Each showed several major peaks of waxes with retention times of 4–9 min (Figure 1A) and several minor peaks with retention times of 9–12.5 min (some sterol esters). The remaining mass, representing approximately 80% of the total quantity injected, was eluted in 15.9–37 min and consisted predominantly of sterol esters as indicated by mass spectral analyses.

The ammonia CI GC-MS total ion monitor trace (Fig. 1B) is quite similar to the capillary GLC. All peaks, when analyzed by ammonia CI, gave $(M + NH_4)^+$ adduct ions as base peak or of high intensity. Esters of phytol and geranylgeraniol were readily identified by ammonia CI mass spectra which exhibited in addition to $(M + NH_4)^+$ adduct ions, $M + NH_4 - RCO_2H$ fragments at *m/z* 296 and 290 which are, coincidentally, equivalent to the molecular weights of phytol and geranylgeraniol, respectively. The $(M + NH_4)^+$ adductions and the $(M + NH_4 - RCO_2H)^+$ ions of high abundances indirectly indicate the molecular weights of the ester, phytol, geranylgeraniol, and the acid moiety. Additionally, the esters of geranylgeraniol gave $M + H - RCO_2H$ fragments at *m/z* 273.

Thus, even though the corresponding esters of phytol and dihydrophytol were poorly separated by GLC, the phytol esters containing $C_{16}$, $C_{18:1}$, $C_{22}$, and $C_{24}$ acid moieties were identified in peaks 2, 4, 6, and 7, respectively (Figure

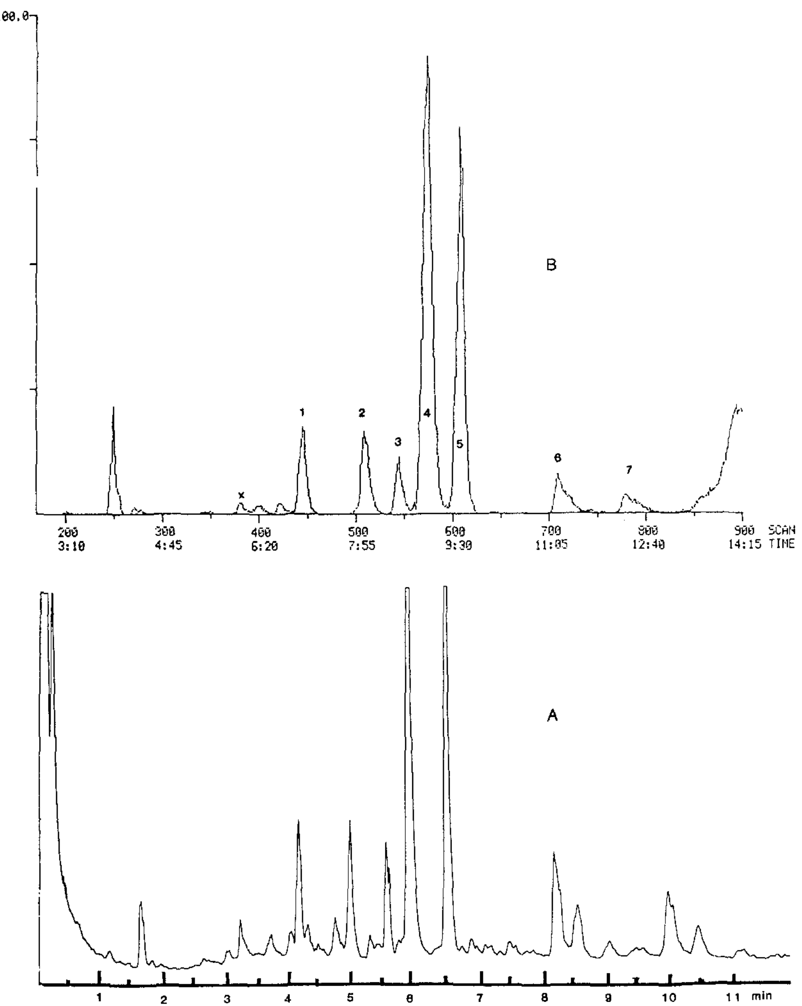


FIG. 1. (A) Capillary GLC trace of the wax region of the wax–sterol ester fraction of cotton anthers; (B) ammonia CI-MS total ion monitor trace of the wax region of the wax–sterol ester fraction of cotton anthers.

1B). They eluted on the back side of these peaks. The molecular weights (by mass spectral analyses) of these esters were 534, 560, 618, and 646, respectively. At the beginning of the front side of the region where some of sterol ester began to elute, the phytol ester (mol wt 674) containing a $C_{26}$ acid moiety was also observed. Since molecular weights of the corresponding esters of dihydrophytol are two mass units higher than those of phytol, the molecular weights of 536 and 562 for the esters eluting on the front side of peaks 2 and 4 suggest

that these are dihydrophytol esters containing $C_{16}$ and $C_{18:1}$ acid moieties, respectively. Similarly, the major esters of peaks 6 and 7 are dihydrophytol esters containing $C_{22}$ and $C_{24}$ saturated acid moieties, respectively. It should be mentioned that saponification of the wax–sterol ester fraction yielded predominantly even-numbered saturated straight chain acids from $C_{12}$ to $C_{28}$. The $C_{14}$ to $C_{28}$ acids were about equally distributed.

Interestingly, the geranylgeraniol esters eluted as peaks of high purity without observable contamination. The adduct $(M + NH_4)^+$ ion, and the $M + NH_4 - RCO_2H$ and $M + H - RCO_2H$ fragments readily identified peaks 3 and 5 as geranylgeraniol esters containing $C_{16}$ and $C_{18:1}$ acid moieties, respectively. The molecular weights of these two esters were 528 and 554.

Other esters were observed in peaks 1, 2, and 4. Ammonia CI GC-MS analyses of peak 1 showed material of a molecular weights of 486 and a small quantity of mol wt of 484; peak 2 showed mol wt of 506 and peak 4 showed mol wt of 532. We could not identify these esters or speculate as to their identity since the alcohol or fatty acid moieties of these peaks could not be determined by EI- or CI-mass spectral analyses. EI mass spectral analyses were of little diagnostic value for the esters.

*Synthetic Esters.* Since our results indicated that the reconversion of the alcohols to esters restored activity, we synthesized and bioassayed a series of fatty acid esters of oleyl alcohol (not necessarily the $C_{18:1}$ alcohol present in anthers or buds), dihydrophytol, phytol, and geranylgeraniol including those found in the wax–sterol ester fraction. The synthetic esters corresponding to those found in the wax mixture from cotton buds or anthers exhibited similar retention times by GLC and gave, by ammonia CI, spectra similar to those of the naturally occurring esters.

In our bioassay, a compound was rated as having high feeding stimulant activity if it induced feeding and caused weevils to produce 50 or more punctures in the treated area of the filter paper than in blank or untreated areas. Our results thus indicate that a number of synthetic phytol and geranylgeraniol esters have high feeding stimulant activity when tested below the 500 $\mu$g level (Table 3). Higher quantities of material on the test paper increased feeding stimulant activity. The phytol ester containing a $C_{12}$ acid moiety was the most active of the phytol esters tested. Yet, GC-MS analyses (Figure 1B, peak X) indicate that this ester is present only in a small quantity in cotton buds or anthers.

Peak 5 (Figure 1B), which was identified as a geranylgeraniol ester containing a $C_{18:1}$ acid moiety and subsequently identified as geranylgeraniol oleate, is a major component of the waxes of cotton buds and anthers. The synthetic geranylgeraniol oleate, however, did not exhibit high feeding stimulant activity.

The dihydrophytol esters containing the $C_{14}$ and $C_{16}$ acid moieties were prepared and tested, and they were inactive. Of the oleyl alcohol esters tested, only the ester containing a $C_{22}$ acid moiety showed high activity. Eicosanol ester with a $C_{14}$ acid moiety was inactive in the feeding stimulant bioassay. Similarly,

TABLE 3. FEEDING RESPONSE OF BOLL WEEVILS PRESENTED SYNTHETIC ESTERS[a]

| Alcohol moiety | Acid moiety | Feeding response |
|---|---|---|
| Geranylgeraniol | $C_{12}$ | 32 |
| | $C_{14}$ | 16 |
| | $C_{16}$ | 31 |
| | $C_{18}$ | −4 |
| | $C_{18:1}$ | 12 |
| | $C_{20}$ | 75 |
| | $C_{22}$ | 110 |
| Phytol | $C_{10}$ | 15 |
| | $C_{12}$ | 110 |
| | $C_{14}$ | 53 |
| | $C_{16}$ | 36 |
| | $C_{18}$ | 73 |
| | $C_{18:1}$ | 32 |
| | $C_{20}$ | 59 |
| | $C_{22}$ | 54 |
| | $C_{26}$ | 59 |

[a]The quantities presented in application of 20 μl of 0.04 M solution ranged from 378 to 490 μg and 361 to 540 μg for the esters of geranylgeraniol and phytol, respectively.

farnesol with a $C_{18}$ acid moiety was inactive. The results suggest that an allylic diterpene alcohol moiety is a prerequisite for an ester to be active, although phytol phytanoate was inactive. Increasing the chain length of the geranylgeraniol esters appears to enhance activity.

A mixture of synthetic esters of phytol and geranylgeraniol found in the wax–sterol fractions of buds and anthers did not show activity higher than that of the most active component present in the mixture. Certain of the synthetic esters, for example the phytol esters containing $C_{12}$, $C_{18}$, and $C_{26}$ acid moieties, were more active than the wax–sterol ester fraction of anthers or buds when tested at concentrations approximating that of the waxes in the extracts. For example, 20 μl of 0.005 M solution or 56 μg of the phytol ester with the $C_{18}$ acid moiety elicited a feeding response of 80 punctures compared to three punctures in the blank. The ratio of response by weevils presented 1.75 μg and 0.877 μg of this ester to blank was 19 : 5 and 26 : 6, respectively. Thus, the low threshold level of feeding response is below 1 μg for this compound.

Host-plant selection by phytophagous insects in early infestation stages is often partially dependent on plant surface stimuli, e.g., stimuli that influence aphids while walking and probing, and beetles during the first probe (Klingauf et al., 1978). The alkane fraction of the host plant promoted pea aphid, *Acyrthosiphon pisum*, movement from the upper to the lower leaf side. It has been

shown that, at least in many cases, the chemical nature of the surface is important in selections. The outer surfaces of the leaf of all terrestrial angiosperms and gymnosperms are covered by a cuticle some 1–13 μm thick. The commonest components are *n*-alkanes, *n*-primary alcohols, fatty acids, and wax esters (Chapman, 1977), although many other chemicals are recorded as minor constituents. The alcohols, *n*-hexacosanol and *n*-octacosanol, have been recently identified from leaves of the mulberry as potent feeding stimulants for larvae of the silkworm, *Bombyx mori* (Mori, 1982). Because *n*-tetracosanol and *n*-triacontanol are also present in mulberry leaves but did not stimulate larval feeding, the author concluded that the chemoreceptors for food selection of the silkworm are precise in recognizing the carbon skeleton of chemicals.

The fact that many esters of phytol and geranylgeraniol have such high feeding stimulant activity certainly suggests that these compounds in cotton buds and anthers are the major feeding stimulants for the cotton boll weevil. The identical carbon chain lengths of phytol and geranylgeraniol also suggest that the chemoreceptor of the boll weevil may be stimulated by the carbon skeleton of these alcohols. We do need to determine, however, the structure of the $C_{18:1}$ alcohol derived from the waxes, and whether the sterol esters present in the wax–sterol fraction enhance the feeding activity of the long-chain esters of phytol and geranylgeraniol.

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# VIDEO CAMERA-COMPUTER TRACKING OF NEMATODE *Caenorhabditis elegans* TO RECORD BEHAVIORAL RESPONSES

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**Abstract**—A new method is used to analyze responses to changes in the concentration of two chemical stimuli. Nematodes are allowed to move around on the surface of a thin layer of agar across which a stream of air blows to carry volatile stimuli. Darkfield illumination provides high-contrast images of the worms which are acquired by a video camera and fed to a microcomputer which is programed to simultaneously track and record the movements and changes in direction of as many as 25 animals. The results are reported in real time. The worms respond to an increase in $CO_2$ concentration by decreasing the number moving and increasing the number of changes of direction. Both responses adapt to steady-state levels in about half a minute. This suggests that they respond by changing the probability of initiating a reversal bout. This observation adds a repellent to the class of stimuli that *C. elegans* reponds to by klinokinesis. The resonses to changes in oxygen concentration are somewhat different. Movements and changes in direction both decrease when the oxygen concentration falls and increase when the concentration rises. No adaptation is seen within the one-minute time span observed. This observation provides further evidence that the response to oxygen differs from the response to other chemicals and may be sensed internally. These observations demonstrate that computer tracking is a sensitive method of analyzing animal behavior. It is further demonstrated that a significant response can be detected to a relatively weak stimulus in less than 5 min.

**Key Words**—*Caenorhabditis elegans*, nematode, chemotaxis, video camera, microcomputer, tracking, carbon dioxide, oxygen, klinokinesis.

## INTRODUCTION

Nematodes are an important group of animals. They are extremely common in most environments and play important ecological roles (Yeates, 1981; Anderson



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*et al.*, 1981). They are serious pests in agriculture (Bird and Thomason, 1980) and cause much disease in livestock and humans. In addition, the free-living nematode *Caenorhabditis elegans* has become an important laboratory model for basic biological studies (Zuckerman, 1980).

One of the important questions about nematodes is how they sense their world and find their way around in it. Since most nematodes live in the darkness of soil and have sense organs that appear to be primarily chemosensory, it is generally assumed that chemical stimuli are the primary means by which they locate food, hosts, and mates. However, very little has been established about how nematodes accomplish these tasks (for recent reviews see Dusenbery, 1980c; Prot, 1980). In particular, there is no case in which a nematode has been shown to locate a target by responding to an identified chemical. In fact, little is known about the stimuli used by any organisms in the soil environment.

In order to increase our understanding in this area, it is important to try to identify some of the chemicals used by nematodes to locate targets in the natural environment. A development that would aid in this task is the design of better, more rapid methods of assaying behavioral responses to chemical stimuli. A new technique is described here that makes use of recent developments in electronics. It uses an inexpensive closed-circuit TV camera interfaced to a microcomputer to simultaneously track many individual nematodes as they crawl over a surface and records, in real time, the amount of movement and the number of changes in direction they make.

## METHODS AND MATERIALS

Behavior was recorded by a video camera interfaced to a microcomputer. The details of this system are published elsewhere (Dusenbery, 1985). In brief, the image is digitized into a 240 × 256 array of one intensity bit. Software has been written that simultaneously tracks many worms and determines the position of each, once a second. The present system has sufficient speed that 25 worms can be tracked simultaneously. The software also counts the number of times each worm moves and the number of times each changes its direction of locomotion. These data are summed for each 10-sec interval.

This system requires that the worms be viewed in high contrast. Consequently a darkfield illumination system was developed. The design is illustrated in Figure 1. Light from an ordinary 25-W light bulb, after passing through a heat filter, is reflected by a cylindrical mirror 20 cm in diameter through the arena where the worms are located and at angles such that the light does not enter the camera. Between the light source and the camera is a light trap that prevents light from directly reaching the camera. Only light that is reflected by the mirror and scattered by worms in the arena enters the camera in significant amounts.

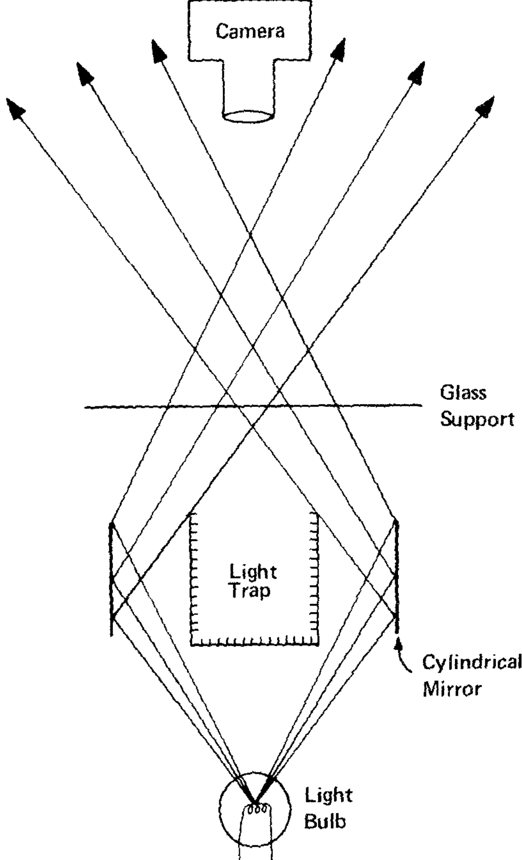


FIG. 1. Diagram of setup for darkfield illumination. The cylindrical mirror was 20 cm in diameter and 8 cm long. Everything in the figure is to scale except the body of the camera.

The worms are placed on a thin layer of agar on a glass slide. The pad of agar is spread out to an area 33 × 40 mm. The slide is then supported above a sheet of glass with a 2-mm-wide air gap between the agar surface and the glass sheet. A stream of air is pumped through this gap in order to carry the chemical stimuli. The usual procedure is to alternate between two stimulus conditions switching at 1-min intervals.

*C. elegans* was originally obtained from S. Brenner and was cultured according to his methods (Brenner, 1974). For this study, young adults were washed off a Petri dish and rinsed in 0.3% NaCl plus 25 mM potassium phosphate buffer of pH 6.0. About 30 young adults were selected and transferred to the agar pool

in approximately 5 $\mu$l of solution. The agar pool was formed with 0.7 ml of 1% agar with the same salt concentrations as the rinsing solution.

## RESULTS

In this study the position of each nematode was determined about once a second. After the new position of each was determined, it was compared to the old position to determine if the individual had moved. If so, this was recorded as a movement. The directions of the movement along the $x$ and $y$ axes were compared to directions of the previous movements however long ago they occurred. If there was a change in direction in both the $x$ and $y$ axes or there was a change in direction in one and no movement in the other, a turn was recorded. This strategy was adopted in order not to count a gradual curve as a turn.

The number of movements and turns recorded were summed for each 10-sec interval. For each stimulus cycle (1 min on, 1 min off), the total number of movements and turns was determined and the fraction of each that occurred in each 10-sec interval was determined. These fractions were then averaged over many stimulus cycles.

Since chemical stimuli must be carried in a stream of air, they are restricted to volatile chemicals. Consequently carbon dioxide and oxygen were selected as stimuli. The former is commonly found to be a stimulus for a variety of nematodes (reviewed by Prot, 1980) and appears to act as a typical chemical repellent for *C. elegans* (Dusenbery, 1974). Oxygen, on the other hand, is an attractant that appears to act through a different receptor mechanism (Dusenbery, 1980b).

The results for carbon dioxide are shown in Figure 2. It is seen that, a few seconds after the $CO_2$ concentration increases, the frequency of movements decreases and then returns to the previous value in about half a minute. In contrast, the frequency of turns increases when $CO_2$ is increased and then adapts in about the same period. The increase in turning peaks later than the decrease in moving. When the $CO_2$ switches back to ambient levels, relatively little change in behavior is observed.

The response to a change in oxygen concentration is somewhat different, as shown in Figure 3. When the concentration increases, the frequencies of both movements and turns decline. When the oxygen concentration decreases, both frequencies increase. In both cases, the change in movements is delayed compared to the change in frequency of turns. These changes take place more slowly than the response to $CO_2$.

There are, thus, several differences in the response to oxygen compared to the response to $CO_2$. The frequency of movements and of turns change in parallel in the case of oxygen stimulation but in opposite directions in the case of $CO_2$. In addition, the initial response to $CO_2$ occurs much more rapidly than

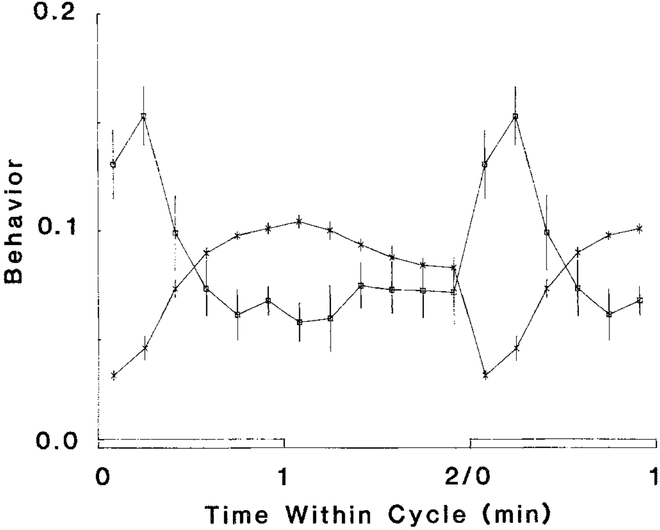


FIG. 2. Behavioral responses to changes in $CO_2$ concentration. The fraction of movements (×) or changes of direction (□) occurring at various times during the stimulus cycle is plotted. The first half of the stimulus cycle on the left is repeated on the right side in order for the changes to be more easily seen. The error bars represent standard deviations for five mean values, each derived from ten continuously repeating stimulus cycles. The bar at the bottom represents the presence of 4% $CO_2$ in a flow of air.

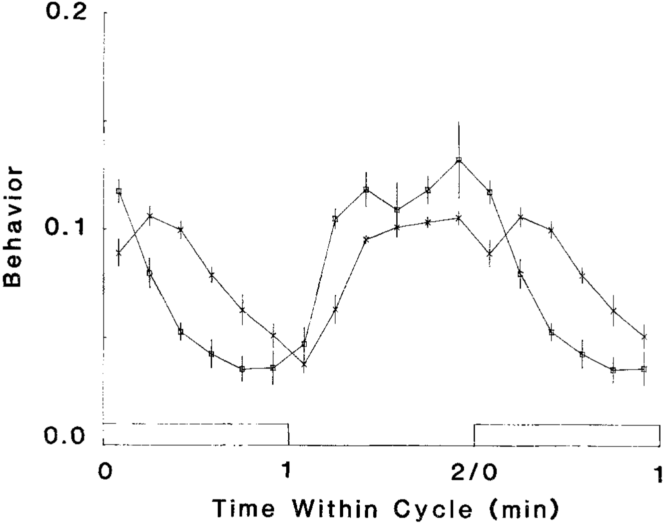


FIG. 3. Behavioral responses to changes in oxygen concentration. The fraction of movements (×) or changes of direction (□) occurring at various times during the stimulus cycle is plotted. The bar at the bottom represents the presence of 10% oxygen in a flow of nitrogen. Other aspects are the same as Figure 2.

that to oxygen. Half the maximal value is reached in less than 10 sec for $CO_2$ compared to 20–30 sec for oxygen.

The previous experiments include data averaged over many hours of testing. In order to assess the value of this technique for screening chemical fractions, it is necessary to determine how rapidly it can make a determination that a stimulus is present. This was done by developing a measure of the strength of the response applicable to a single stimulus cycle. The measure that was adopted was the normalized difference between the halves of the stimulus cycle in reversals in the last 50 sec of each half of the stimulus cycle. Specifically, the response is 100 × [No. reversals (stimulus off) − No. reversals (stimulus on)]/[No. reversals (stimulus off) + No. reversals (stimulus on)]. This provides a measure which varies between plus and minus 100 and is 0 if there is no response. This measure was calculated for a subset of the data presented in Figure 3 in which the worms were exposed to oxygen stimulation and for data from a control experiment in which air flowed in both halves of the stimulus cycle. The distribution of response scores is presented in Figure 4. The scores from the control experiment are centered around zero with a spread of approximately 10 units on each side. The responses from the oxygen stimulus experiment are centered around 20 with some overlap of the control scores.

## DISCUSSION

The normal sequence of behaviors seen when *C. elegans* responds to a sharp change in stimulation is that it stops moving forward, pauses for a few

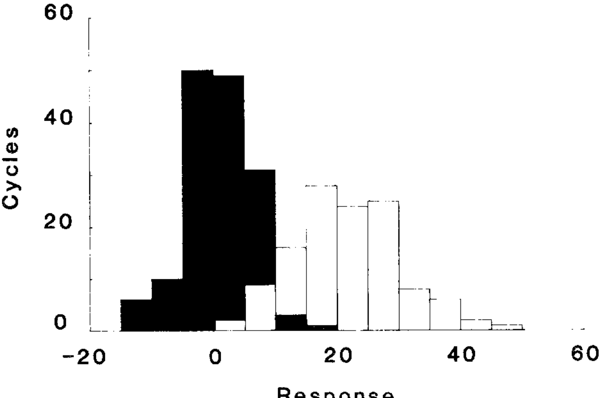


FIG. 4. Distribution of response scores for single cycles of stimulation. The filled bars represent data from control experiments in which no stimulus was present. The open bars represent data from experiments where the stimulus was 10% oxygen.

seconds, backs up a few wavelengths, turns, and goes off in a new direction (Croll, 1975; Dusenbery, 1980a). This behavioral sequence is called a reversal bout. When such a behavioral sequence is analyzed by the tracking program employed here, one would expect to observe a decrease in frequency of movements followed by an increase in frequency of turns. If all the individuals being tracked perform this behavior simultaneously, clearly defined peaks and valleys in the responses should be observed. This is just what is found in the case of $CO_2$. This indicates that the worms are responding to the repellent $CO_2$ by performing a standard reversal bout and that most of them do it immediately after stimulation.

A comparison of the pattern of the responses to $CO_2$ and the pattern of reversal bouts resulting from changes in NaCl stimulation recorded by the tether technique (Dusenbery, 1980a) indicates some differences. Perhaps the most notable is that with NaCl there was both an increase in reversal bouts when the concentration changed in one direction and a decrease in reversal bouts when the concentration changed in the other direction. In the experiments reported here, the response to $CO_2$ was primarily limited to the increase in concentration. It is not clear why this difference occurs.

The response of *C. elegans* to oxygen has previously been reported to differ from the responses to other chemicals in certain respects. The principal observation is that mutant strains that had anatomical abnormalities in all the apparent chemoreceptor organs on the head still made normal behavioral responses to changes in oxygen (Dusenbery, 1980b). This led to the supposition that the oxygen tension is sensed internally. If this is correct, one might expect significant differences in the response to this stimulus compared to others. In particular, the response might be slower because of the requirement that the stimulus chemical diffuse through the cuticle to reach the internal receptor. A slower response is, in fact, what is observed. In addition, the response is different in character with movements and changes in direction moving in parallel. This provides further evidence that the response to oxygen is distinctly different and supports the hypothesis that it is sensed internally.

The method employed in this study offers some distinct advantages over previous methods of analyzing nematode behavior. The primary one is that behavioral data are acquired rapidly and are immediately available. This is particularly advantageous in the study of relatively weak stimuli such as oxygen. The technique previously used with oxygen (Dusenbery, 1980b) was to tether individual nematodes in a flowing stream of water which carried the stimulus. The tether technique required many hours to obtain statistically significant data on the week response to oxygen. In contrast, the computer-tracking technique described here permits the detection of a response in less than 5 min. This difference is primarily due to the fact that the tether records data from only one animal at a time, while the computer can track 25. Video methods have previously been used to study nematode behavior. However, they were relatively slow as the

video images were recorded and later analyzed by hand (Croll, 1975). A computer system to analyze the behavior of another type of small invertebrate has been developed, but it works on video tapes rather than in real time (Miller et al., 1982).

The restriction to volatile stimuli may seem to be a serious problem. However, there are good reasons to think that the most important chemical stimuli in the soil environment will turn out to be volatile (Bone and Shorey, 1978). The principal reason is that diffusion through air is about 10,000 times faster than through water. Thus volatile stimuli can establish chemical gradients in the stagnant air of soil much more rapidly than nonvolatiles. For example, the best stimuli for zoospores of certain plant pathogenic fungi that are found in soil are volatile (Cameron and Carlile, 1978). The fact that active nematodes are covered by a film of water does not impose much of a limitation, since most chemoreceptors are similarly covered. Another consideration is that most of the methods previously employed to measure nematode responses to chemicals would have lost volatile stimuli.

The main objective in developing this technique was to use it as a rapid assay in fractionating and identifying chemical stimuli from the natural environment. For this purpose, it is important to determine how quickly it can produce a reliable indication of whether a response occurred. From the data in Figure 4, it can be calculated that if the criterion were adopted that a score above 10 constituted a significant response and a lower score indicated no response, then the error rate would be 3% false positives and 6% false negatives. Alternatively, if the criterion were adopted that scores between 5 and 15 were indeterminate, then the error level would be about 1% with about 20% of tests indeterminate. This is very promising for one 2-min test in which the results are immediately available.

Once the nematodes are placed in the apparatus, they remain active for many hours. Thus, many tests can be run one after the other. With the results immediately available, important or ambiguous results can be immediately confirmed by retesting. This permits several rounds of testing and fractionation in a single day. In addition, it may prove possible to connect this test apparatus directly to the effluent of a gas chromatograph, and identify active fractions immediately.

The technique described here is also likely to be useful in the study of the behavior of other types of animals. Its principal limitation is that the animals must be visible in high contrast with respect to the background. This criterion could be met in certain circumstances by organisms ranging from bacteria to humans and is likely to be relaxed as technology improves.

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# BEHAVIOR OF THE WESTERN PINE BEETLE DURING HOST COLONIZATION[1]

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**Abstract**—After living ponderosa pines were baited with either female-infested bolts or synthetic pheromones, *Dendroctonus brevicomis* were caught on sticky screens throughout trapping periods of 15–46 days; however, large numbers of beetles were trapped during only a small portion (5–10 days) of these trapping periods. The most attractive portions of trees attacked contained 3–6 beetles $dm^2$, in galleries ca. 2 cm long. Catch increased following addition of males to female-infested bolts, supporting the hypothesis that male-produced frontalin is an attractive pheromone of the western pine beetle. Catch at bolts removed from trees under attack was strongly dependent upon levels of boring activity. We found no evidence of interruption of the response to attractants during host colonization.

**Key Words**—*Pinus ponderosa*, *Dendroctonus brevicomis*, pheromone, attractant, interruptant, *exo*-brevicomin, frontalin, Coleoptera, Scolytidae.

## INTRODUCTION

The western pine beetle, *Dendroctonus brevicomis* LeConte, is a major cause of mortality of pondersa pine, *Pinus ponderosa* Dougl. ex. Laws., in western North America (Miller and Keen, 1960). Beetles are attracted by pheromones

[1] Coleoptera: Scolytidae. Trade names and commercial enterprises or products are mentioned solely for information and do not constitute endorsement by the U.S. Department of Agriculture or University of California.



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released during excavation of entrance tunnels under the bark (Wood, 1972; Wood and Bedard, 1977). Mating, oviposition, and brood development proceed after death of the tree. Wood and Bedard (1977) defined the concentration phase of host colonization as beginning when the beetles start releasing attractants, and the establishment phase as beginning when they start ovipositing.

Females boring in freshly cut ponderosa pine logs are a source of attractant (Vité and Gara, 1962). Furthermore, female-infested ponderosa pine bark and wood are attractive to males in the laboratory (Wood and Bushing, 1963) and to both sexes in the field (Bedard et al., 1969). A pheromone, *exo*-brevicomin, and a terpene of host origin, myrcene, were identified from frass produced by females boring in ponderosa pine (Silverstein et al., 1968; Silverstein, 1970). The mixture of these two compounds is weakly attractive in the field (Bedard et al., 1969, 1980b). Kinzer et al. (1969) and Pitman et al. (1969) identified frontalin, an analog of *exo*-brevicomin, from hindguts of male *D. brevicomis.* The mixture of *exo*-brevicomin and frontalin, plus myrcene (Bedard et al., 1970, 1980b) or turpentine from the host (Vité and Pitman, 1969; Bedard et al., 1980b) is highly attractive in the field. These results refuted earlier proposals (Vité and Gara, 1962; Vité and Pitman, 1968) that the aggregation pheromone is produced by the sex which initiates the entrance tunnel.

Renwick and Vité (1970) proposed a behavioral sequence for *D. brevicomis* during host colonization in which females initially release both frontalin and *exo*-brevicomin prior to feeding on the host. Subsequently, females produce primarily *exo*-brevicomin, while males produce frontalin and verbenone. The combination of the attractants, *exo*-brevicomin and frontalin, and the "inhibitor," verbenone, causes the focus of attraction and attack to shift to nearby trees. However, Libbey et al. (1974) reported that unfed, newly emerged *D. brevicomis* released *exo*-brevicomin and frontalin when both sexes were present. No frontalin was released when only females were present. Browne et al. (1979) found that air passing over bolts infested with *D. brevicomis* females contained *exo*-brevicomin and myrcene, and that when males were allowed to join the females, frontalin was released.

We undertook the studies reported herein in order to clarify aspects of host colonization: (1) the sequence of arrivals at attacked trees, (2) the attractiveness of infested host material during host colonization, (3) possible interruptive effects of volatiles released late in the host colonization process, and (4) the respective roles of females and males in creating the attraction. Wood and Bedard (1977) reported some conclusions drawn from these studies without presenting methods, data, or analyses.

## METHODS AND MATERIALS

Four studies were conducted in Madera County, in east central California.

*Arrival Sequence at Trees.* The sequence of arrivals of *D. brevicomis* dur-

ing colonization was monitored on 12 trees near Bass Lake, July–September 1969 and July–August 1970. Individual traps, 30.5- × 61-cm flat sticky screens of 0.95-cm mesh hardware cloth coated on both sides with Stikem Special®, were affixed to the bole of each tree (Bedard et al., 1969) at 3, 6, and 9 m above ground. Colonization was induced by affixing to the bole at 6 m above ground either a ponderosa pine bolt with 100 *D. brevicomis* females in preformed entrance tunnels or a device (Tilden et al., 1981) that released racemic *exo*-brevicomin, racemic frontalin, and myrcene at ca. 2 mg/24 hr (each compound).

The concentration phase of colonization was considered to be underway and the bait was removed when at least two of the three traps on a tree caught 50 or more beetles during a single day. This rule was chosen on the basis of previous experience with baited trees killed by *D. brevicomis*. Beetles were picked from traps daily, beginning the day after bait placement and continuing until the 17th day after bait removal. Beetles were placed in labeled vials of solvent and taken to the laboratory where they were counted and their sex was determined. When the daily catch of *D. brevicomis* on a trap exceeded 50, a random sample of 50 beetles was selected for sex determination.

Numbers of *D. brevicomis* caught were graphed to reveal any consistent patterns in the arrival sequence. Catch on each of the three traps and the total catch on all three traps were plotted against time (number of days after bait placement), independently for each tree. Three-point running averages of the daily catches were used to reduce the effects of random variation and make any consistent patterns more apparent.

Shifts in the sex ratio of attacking beetles during colonization were investigated by pooling the data from all colonized trees and computing the proportion of male beetles in the total daily catch for each of the 17 days of trapping following bait removal. All 17 proportions were simultaneously tested for equality to 0.5 (a sex ratio of 1 : 1) by applying the standard normal approximation for a binomial proportion (Johnson and Kotz, 1969) and using a maximum modulus test (Miller, 1966) of size (alpha) 0.05. The linear trend of the male proportion was estimated by performing a linear regression analysis of the daily proportions against time.

*Attractiveness of Bolts and Concomitant Activity of* D. brevicomis. Colonization of trees was induced with female-infested bolts during July to September 1969 or the attractive mixture described above during July and August 1970. Attractant-producing substrates were affixed to the tree bole 6 m above ground. Beetle arrival was monitored at 3, 6, and 9 m above ground with flat, sticky traps, as described above. Trees being colonized were felled 2–13 days after baiting. Pitch tubes, boring dust, sound of boring, stridulation, and destructive sampling to determine gallery length were used to assess progress of colonization. Felling dates were chosen to provide observations throughout colonization.

Three 61-cm bolts were cut from the bole, one at each trap height, and bioassayed for attractiveness. Bolts were wrapped in wire mesh (1.6 × 1.4 mm)

insect screen, to prevent ingress or egress of beetles, and placed on plywood on top of a metal garbage can. A cylindrical trap (61 cm high × 50 cm diam) of hardware cloth (0.95 cm mesh) coated with Stikem Special was placed around the bolt. Bolts and cylindrical traps were placed ca. 40 m apart in ca. 90-year-old pine stands near Bass Lake and Cedar Valley. Bolts were randomly assigned to test positions each morning. Beetles were removed from the traps the following morning and counted and the sex determined in the laboratory. After 3–6 days exposure, bolts were dissected to determine the number and sex of beetles in each.

Adjacent bolts (30.5 cm long) on either side of the bolt to be assayed were cut and dissected in the laboratory to determine the number and lengths of galleries and the number and sex of beetles present. These data were taken to assess the conditions in the assay bolts at the beginning of the experiment.

*Interruptive Effect of Bolts from Trees in Establishment Phase.* Bolts from trees in the establishment phase were placed with bolts from trees in the concentration phase to determine their possible interruptive effect (Wood, 1977) on beetle response during August and September 1970, near Cedar Valley. Attack was induced with the synthetic attractants, and arrival was monitored, as in the arrival sequence study. In one experiment, a tree in the establishment phase was cut 8 days after peak arrival and assayed for 5 days. In a second experiment, a tree in the establishment phase was cut 20 days after peak arrival and assayed for 4 days. In both experiments, trees in the concentration phase were cut at apparent peak arrival, 4 days after baiting. The central bole of each tree was cut into 16 bolts, each 30.5 cm long. Each bolt was wrapped with fine mesh (1.6 × 1.4 mm) screen.

Eight test positions were installed in two rows perpendicular to the prevailing winds and ca. 400 m apart within a ca. 90-year-old pine stand. Each row consisted of four positions, 30 m apart. On the first day, two bolts from the concentration-phase tree were assigned to each position and two randomly chosen bolts from an establishment-phase tree were assigned randomly to every other position. On each successive day until the end of an experiment, the two bolts from the establishment-phase tree were moved to the adjacent position, which on the previous day had bolts only from the concentration-phase tree. Establishment-phase bolts were moved each day by 0900 PDST, and beetles were removed from the traps and then sex determined. Two bolts were used to assure a strong attractive source. We assumed that concentration-phase bolts taken from the bole nearer to the bait had been colonized earlier and therefore were more attractive than bolts taken further from the bait. Each pair consisted of a bolt taken near the bait and one taken farther from the bait.

Cylindrical sticky traps, as described above, were supported on two strips of wood across the open top of a garbage can. When four bolts were present, they were stacked on top of one another with the lowest bolt resting on the bottom of the can. When only two bolts were present, they were stacked on top

of one another on top an inverted 61-cm-high metal can within the garbage can. The height of the top bolt, therefore, was the same at each test position.

Each day's catches were analyzed as a block of data. Catches were compared between traps within blocks to reduce effects of daily differences. We ignored trap lines, treating as eight positions, rather than as two sets of four positions. Thus, each block contained catches from four traps with bolts from the concentration phase (control) and from four traps with bolts from the concentration and establishment phases (treatment). Catch at the treatment was compared with catch at the control by a distribution-free, randomized-blocks analysis with aligned ranks (Mehra and Sarangi, 1967). Alignment consisted of dividing each catch by the total for the block.

The catch data were also analyzed for a possible shift in male proportion caused by bolts from the establishment phase. Each day's data were summarized in a $2 \times 2$ contingency table showing total male catch vs. total female catch on one axis and catch at treatment bolts vs. catch at control bolts on the other. The male proportions of total catch at treatment traps and at control traps for each day were determined, and the difference between them calculated. The differences were analyzed using the Mantel-Haenszel procedure (Fleiss, 1973).

*Roles of Sexes in Attraction.* The roles of males and females in attraction were measured by comparing catches at four kinds of bolts: (1) uninfested, (2) infested with 100 males, (3) infested with 100 females, and (4) infested with 100 females with 100 males added 2–3 days later. Bolts (61 cm long × 20–26 cm diam) were cut from living pines and their cut ends were dipped in hot paraffin to retard moisture loss. Individual beetles were confined inside preformed tunnels with fine mesh screen. Tunnels were made by driving a blunt rod into the inner bark. Infested bolts were stored in the dark overnight to encourage boring. Before the bolts were taken to the field, the confining screens were removed, and the bolts were loosely wrapped in fine mesh screen to prevent the entrance of attracted beetles.

In an attempt to prolong the period when bolts would be attractive, ca. 200 ml of water were added to each bolt daily during field exposure. Water was supplied daily from a 250-ml plastic bottle fitted to an iron pipe driven into the sapwood at one end of the bolt.

The relative attractiveness of bolts was assayed by supporting cylindrical traps on plywood on top of metal garbage cans as described above. In August 1969, near Bass Lake, eight traps were placed in two lines ca. 300 m apart and perpendicular to the prevailing winds. The four traps in each line were at least 20 m apart. One bolt of each of the four treatments was randomly assigned to a position in each line and set out before 1200 hr. Between 1600 and 1700 hr the following day, trapped beetles were removed from the screens, counted, and then sex determined. This test was repeated in September. The two tests were analyzed as a single experiment with four replicates of each treatment. The males were added to the female-infested bolts between 1600 and 1700 hr of the

second day of the August test and on the third day of the September test. The eight bolts used in each test were cut from one living pine no more than one week before use.

Analyses of data from this experiment consisted of comparisons based on catch at female-infested bolts (control) and female-infested bolts to which males were added (treatment). Only two trapping periods were of interest: (1) the 24 hr before and (2) the 24 hr after addition of males. Catch before addition of males was subtracted from catch after addition, giving the increase in catch. This operation was also applied to catch at the control bolts, even though the source of attraction—100 females—was unchanged. Within each of the two groups (control and treatment) of four bolts, one-sided Wilcoxon signed-rank tests were used to determine, for control and treatment separately, if the increase in catch was significant. Tests of size (alpha) 0.0625 rather than 0.05 were made, because the smallest significance probability possible for a sample of size four is $(\frac{1}{2})^4 = 0.0625$. The power of these two tests must have been low because of the small sample size. The Wilcoxon rank-sum test was used to test whether the increase in catch at the treatment traps was equal to the increase at the control traps. This was also a one-sided test, the alternative being that the increase at the treatment traps was greater than the increase at the control traps.

Possible changes in the proportion of male beetles caught were tested with the Mantel-Haenszel procedure, in which the catch data from each bolt were analyzed as a $2 \times 2$ contingency table. The change in sex ratio at each trap was expressed as the difference between the proportions of male beetles caught before and after males were added. Again, this procedure was applied to catch at control bolts, even though the source of attraction was unchanged.

## RESULTS

*Arrival Sequence on Trees.* Of the 12 baited trees, seven were colonized and killed. Day-to-day variation in catch at these seven trees was large. The only consistent pattern in catches among the seven trees was a 5- to 10-day period when catch was markedly higher than that during any other period (for example, see Figure 1).

The proportion of males in the total catch for all colonized trees, at all heights, over all days, wasd 0.4955. The proportion of males in the daily catch did not differ significantly from 0.5 for any of the 17 days of trapping (simultaneous alpha = 0.05). The slope of the regression of male proportion on day of catch was 0.0008 and did not differ significantly from zero [$P$ (2-sided) = 0.5319]. The intercept was 0.4893, which did not differ significantly from 0.5 [$P$ (2-sided) = 0.2929].

*Attractiveness of Bolts and Concomitant Activity of D. brevicomis.* At bolts containing galleries that were mostly shorter than 2 cm (inferred from gallery

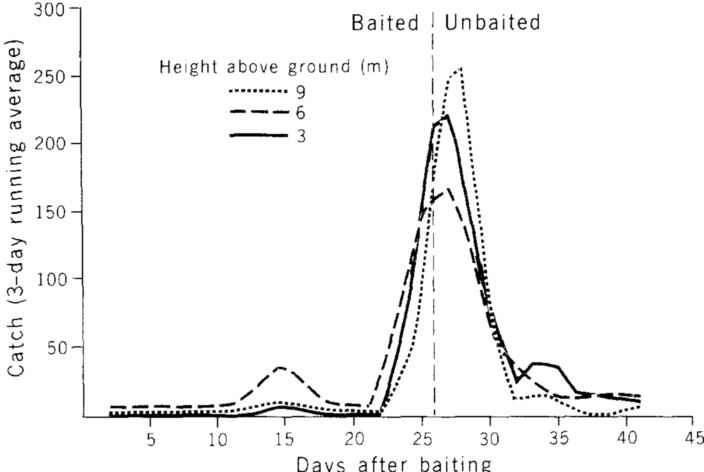


FIG. 1. Numbers of *D. brevicomis* caught on sticky screens at three heights on the bole of a tree baited with a female-infested bolt, Madera County, California, July and August, 1969. (Catch appears high for day of bait removal because 3-day running average is used. Value shown is average of catch on day before, day of, and day after bait removal.)

lengths in adjacent bolts), more beetles were caught where beetle density was higher (Table 1, trees 1-69, 3-69, and 1-70, IV, V, and VI). Traps at bolts with low beetle density (i.e., 1-69, 9 m; 2-69, 3 m and 9 m; 1-70, 9 m) caught disproportionately fewer beetles after the bolts were excised from the bole than before. For example, the bolt from the 9-m height on tree 1-69 caught less than 1% of the combined total catch of all bolts from this tree (two out of 594, Table 1, IV). By comparison, the trap at the 9-m height, before the tree was cut, caught 26% of the combined total catch from all heights (81 out of 310, III).

Males had joined females in 54% of the galleries in adjacent bolts ($N$ = 235). In 25% of the galleries less than 2 cm long, beetles had not penetrated to the inner phloem, yet males already were present behind females in about half of them.

*Interruptive Effect of Bolts from Trees in Establishment Phase.* Catch at traps baited with bolts from concentration- and establishment-phase trees (treatment) was not significantly different from catch at traps baited with bolts from concentration-phase trees only (control). When bolts from establishment-phase trees were cut 8 days after peak arrival, the randomized blocks analysis gave a significance probability ($P$ value) of 0.829. When bolts from establishment-phase trees were cut 20 days after peak arrival, the significance probability was 0.796. In the first experiment, the mean catch at treatment bolts was 5.31 (SE = 0.47), while mean catch at control traps was 5.69 (SE = 0.75). In the second experiment, mean catch at treatment bolts was 17.15 (SE = 2.71) and at control bolts

TABLE 1. CATCH OF *Dendroctonus brevicomis* ON TRAPS ON TREES AND AROUND BOLTS TAKEN FROM TREES, AND EXTENT OF BORING ACTIVITY IN PORTIONS OF TREES UNDERGOING COLONIZATION FOLLOWING BAITING,[a] MATERA COUNTY, CALIFORNIA, JULY–SEPTEMBER 1969, JULY–AUGUST 1970

| Tree number I | Height (m) above ground II | Total catch[b] At tree III | At bolt IV | Number of beetles per $dm^2$[c] V | Frequency of gallery length (cm)[d] <2 VI | 2-5 VII | >5 VIII |
|---|---|---|---|---|---|---|---|
| 1-69 | 9 | 81 | 2 | 0.19 | most | | |
| 1-69 | 6 | 117 | 448 | 3.83 | most | | |
| 1-69 | 3 | 112 | 144 | 2.39 | most | | |
| 2-69 | 9 | 26 | 5 | 0.01 | —[e] | | |
| 2-69 | 6 | 245 | 736 | 2.48 | — | | |
| 2-69 | 3 | 105 | 91 | 0.54 | — | | |
| 3-69 | 9 | 81 | 242 | 1.01 | all | | |
| 3-69 | 6 | 339 | 333 | 4.36 | most | few | |
| 3-69 | 3 | 246 | 632 | 5.21 | most | | |
| 1-70 | 9 | 126 | 9 | 0.37 | most | | |
| 1-70 | 6 | 363 | 183 | 2.48 | <half | >half | few |
| 1-70 | 3 | 369 | 234 | 2.80 | half | <half | few |
| 2-70 | 6 | 663 | 32 | 4.57 | 6 | 3 | 32 |
| 2-70 | 3 | 753 | 42 | 6.05 | 4 | 4 | 39 |
| 3-70 | 6 | 353 | 108 | 2.65 | 12 | 10 | 0 |
| 3-70 | 3 | 209 | 228 | 1.27 | 8 | 3 | 0 |

[a] Colonization was initiated by baiting the bole of each tree at 6 m above ground with female-infested bolts (1969) or the mixture of racemic *exo*-brevicomin, racemic frontalin, and myrcene, each released at 2 mg/24 hr (1970).
[b] Comparisons between catches are valid for simultaneous comparisons only, i.e., between lines.
[c] Determined from assay bolts after assay.
[d] Measured in bolts above and below assay bolt when assay was initiated.
[e] Not dissected.

was 15.90 (SE = 1.77). In both experiments, the Mantel-Haenszel chi-square tests for shifts in the sex ratio were not significant, indicating that bolts from the establishment phase tree did not affect the sex ratio of beetles trapped.

*Role of Sexes in Attraction.* Uninfested and male-infested bolts generally attracted no beetles and therefore were excluded from the analysis. Total catch at the treatment bolts (female-infested, plus males) increased from 139 during the 24 hr before males were added to 496 during the 24 hr after males were added, while catches at the control bolts (female-infested only) were 189 and 188 during the same periods. Catch at the treatment bolts increased significantly ($P$ = 0.0625, one-sided signed-rank test), whereas the catch at control bolts did not ($P$ = 0.8125, one-sided signed-rank test). Furthermore, the increase at the

treatment bolts was significantly greater than the increase at control bolts ($P$ = 0.0143, one-sided rank-sum test).

The addition of males to female-infested bolts also affected the proportion of male beetles trapped. Before addition of males, the average proportions of males caught were 0.59 for control and 0.71 for treatment. After addition of males, the average proportions of males were 0.57 (control) and 0.47 (treatment). The Mantel-Haenszel chi-square (association) was significant ($P$ = 0.0091), indicating that the average difference (over all bolts) between pre- and posttreatment proportions of males was not zero. However, the chi-squares (homogeneity) was also significant ($P$ = 0.0109), indicating that the difference between pre- and posttreatment proportions of male catch varied significantly among the eight bolts. Consequently, the chi square (homogeneity) was partitioned into three component chi-squares: (1) a chi-square (homogeneity) for catch at the four control bolts (significant, $P$ = 0.0116); (2) a chi-square (homogeneity) for catch at the four treated bolts (not significant, $P$ = 0.5752); and (3) a chi-square comparing the average difference between pre- and posttreatment proportions of male catch at control bolts to the average difference at treated bolts (significant, $P$ = 0.0222). These results indicate that the treated bolts showed consistent changes in the proportion of males, with the above-noted variation among the eight bolts being the result of two phenomena: First, the average change in the proportion of males caught at control bolts differed from the average change at treated bolts; and second, the change in the proportion of males caught varied significantly among the four control bolts. This latter phenomenon is difficult to interpret because the source of attraction was unchanged in the control bolts.

## DISCUSSION

The pattern of a 5- to 10-day period of high catch of *D. brevicomis* at baited trees during colonization is similar to the "mass attack" phenomenon described for *Ips paraconfusus* Lanier (Wood and Vité, 1961) and *D. frontalis* Zimmerman (Coster et al., 1977), and "mass arrival" for *D. brevicomis* (Stephen and Dahlsten, 1976). However, by baiting trees and by setting an arbitrary threshold (two traps catching at least 50 beetles each in one day) in order for trees to be included in the study, we may have biased the results, i.e., limited the possibility that other patterns would be detected. This limitation associated with baiting may also apply to the studies by Stephen and Dahlsten (1976), in which trees were baited with either synthetic pheromone or female-infested bolts, and by Coster et al. (1977), in which trees were baited with female-infested bolts.

Our results appear to differ from those of Stephen and Dahlsten (1976) in three aspects. First, the proportion of males trapped in our study during the concentration phase was very nearly 0.5, but they trapped more males than fe-

males during the corresponding period. Second, we observed no trend in the proportion of males trapped during the period of colonization studied, whereas they found a significant shift, i.e., males were trapped in higher numbers than were females during concentration and early establishment phases, but by late establishment, males and fmales were trapped in equal numbers. Third, peak arrival periods, which reflected mass attack, were apparently shorter in our study (5–10 days) than in theirs (a mean of 19.4 days) for trees attacked after June. We measured daily catch over the first 17 days after the start of peak arrival, while they measured catch over various periods that averaged 2–3 days, for 63–73 days after baiting. With *D. brevicomis*, the length of peak arrival and attack period depends on the abundance of beetles available to attack: the fewer the beetles, the more protracted the peak arrival period (Miller and Keen, 1960). Gara et al. (1965) reported the same pattern for southern pine beetle, *D. frontalis*. A similar phenomenon could explain the apparent difference in length of the peak arrival period in our study compared with that of Stephen and Dahlsten (1976). The weather would also be expected to infuence arrival rate (Miller and Keen, 1960) and length of the arrival period.

Vité and Pitman (1968) and Renwick and Vité (1970) have postulated an attack mechanism for *D. brevicomis* in which they stressed the release of attractive pheromones before feeding in a new host. Libbey et al. (1974) demonstrated that pheromones could be released by unfed adults under laboratory conditions. If attractive pheromones were released onto the bole by beetles crawling on the bark in our experiment, then the pattern of catch at bolts cut from different heights on an attacked tree should have been similar to the pattern of catch on sticky traps at the trees before they were cut. However, bolts containing low beetle density were only slightly attractive when removed from the tree.

We found no indication of interruption of response to attraction by bolts from trees that had already been attacked, i.e., bolts cut from trees 8 and 20 days after peak arrival. These results do not support the host colonization mechanism proposed by Renwick and Vité (1970) for *D. brevicomis*, in which production of verbenone by males interrupts the response of both sexes to the attractant, thus decreasing the number of new attacks. Furthermore, we have demonstrated that verbenone, which is produced by males, interrupts the response of *D. brevicomis* to naturally produced attractants from trees under attack (Bedard et al., 1980a). Our results and those of Stephen and Dahlsten (1976) show that attraction during the natural colonization process continues well beyond initiation of the establishment phase. However, we do not know whether beetles that arrive during the establishment phase initiate boring on the infested tree.

The role of male-produced verbenone in the host colonization by *D. brevicomis* can be questioned further on the basis of results reported by Browne et al. (1979)—the amount of verbenone recovered from air drawn over a log that contained *D. brevicomis* females did not differ from that drawn over a similar

log containing males and females. Libbey et al. (1974) also reported release of verbenone by both sexes of *D. brevicomis*. Byers and Wood (1980) found verbenone in male *D. brevicomis* dissected form bolts that were attractive to *D. brevicomis* in the field. Elucidation of the role(s) played by male- and female-produced verbenone in host colonization depends on future studies to determine when and how much verbenone is released in relation to the components of the attractive pheromone and what behaviors for each sex (e.g., flying and walking, entering galleries, and boring) are elicited by these mixtures of attractants and interruptants. The multifunctional role of *trans*-verbenol (Bedard et al., 1980a) and the possibility that verbenone is also multifunctional should be considered in such studies.

Our results are consistent with the hypothesis that males contribute to the attractive pheromone. Males were present during periods of high attractiveness, and the addition of males to female-infested bolts increased their attractiveness and changed the male proportion of the attracted beetles. The change in the proportion of males is apparently the result of a greater increase in attraction for females than for males, after addition of males to female-infested bolts.

Kinzer et al. (1969) and Pitman et al. (1969) showed that hindguts of male *D. brevicomis* contain frontalin, and Libbey et al. (1974) demonstrated that *D. brevicomis* males released frontalin. The addition of males to female-infested logs results in the release of frontalin into the air around the logs (Browne et al., 1979). We believe that increased attraction after addition of males to female-infested bolts results from the release of frontalin by the males. Frontalin is known to enhance the attractiveness of the combination of female-produced *exo*-brevicomin and tree-produced myrcene, turpentine (Bedard et al., 1980b), or oleoresin (Vité and Pitman, 1969). Also, frontalin added to *exo*-brevicomin plus oleoresin increases the catch of females more than it does that of males, so that males and females are caught in equal numbers (Vité and Pitman, 1969). In our experiment, both of these frontalin-produced effects were duplicated by the addition of males. These results are consistent with the hypothesis that male-produced frontalin is a component of the *D. brevicomis* pheromone.

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# ($R^*$,$S^*$)-5-HYDROXY-4-METHYL-3-HEPTANONE Male-Produced Aggregation Pheromone of *Sitophilus oryzae* (L.) and *S. zeamais* Motsch.

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**Abstract**—A male-produced aggregation pheromone, common to the rice weevil, *Sitophilus oryzae*, and the maize weevil, *S. zeamais*, was isolated and identified from hexane extracts of highly absorbent paper disks exposed individually to young virgin male weevils. A combination of preparative column and gas–liquid chromatography of disk extracts yielded purified natural pheromone. When analyzed by nuclear magnetic resonance spectroscopy and gas chromatography–mass spectrometry, the structure of the natural pheromone, "sitophilure," proved to be ($R^*$,$S^*$)-5-hydroxy-4-methyl-3-heptanone, of unknown enantiomeric composition. Synthetic racemic pheromone was highly attractive to males and females of both rice and maize weevils. In addition, both sexes of the granary weevil, *S. granarius* (L.) were attracted to the racemic preparation.

**Key Words**—Aggregation pheromone, attractant, ($R^*$,$S^*$)-5-hydroxy-4-methyl-3-heptanone, ($R^*$,$R^*$)-5-hydroxy-4-methyl-3-heptanone, sitophilure, *Sitophilus oryzae*, *S. zeamais*, *S. granarius*, Coleoptera, Curculionidae.

## INTRODUCTION

Weevils of the genus *Sitophilus* cause serious economic losses of stored cereal grains throughout the world. Effective, cost-efficient grain weevil management could be accomplished by monitoring pest populations with pheromone-baited



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insect traps and applying controls only when pest densities reach economic thresholds (Burkholder, 1981). Male-produced aggregation pheromones which attract both sexes were reported for the rice weevil, *Sitophilus oryzae* (L.) (Phillips and Burkholder, 1981), the granary weevil, *S. granarius* (L.) (Faustini et al., 1982), and the maize weevil, *S. zeamais* Motsch. (Walgenbach et al., 1983). Walgenbach et al. (1983) studied the interspecific attraction among the three species. Schmuff et al. (1984) identified a pheromone common to the rice and maize weevils as the $R^*,S^*$ diastereomer of 5-hydroxy-4-methyl-3-heptanone of unknown enantiomeric composition and synthesized the racemate of this compound. This paper reports further on the isolation, purification, chemical identification, and synthesis of the pheromone, as well as its biological activity among the grain-infesting *Sitophilus* species.

## METHODS AND MATERIALS

*Insect Rearing.* Rice, maize, and granary weevils were obtained from laboratory stock cultures and reared on soft spring wheat (Phillips and Burkholder, 1981). At 4 weeks, newly emerged adults were sieved from wheat cultures and sexed by dimorphic rostral characteristics (Halstead, 1963). Groups of 100 males and females were then isolated in separate 118-ml screen-topped bottles, each containing 50 ml of wheat, and held at standard rearing conditions (16 : 18 light–dark photoperiod, 27 ± 1°C, 60% ± 10% relative humidity).

*Pheromone Collection.* Pheromone was obtained by placing 1- or 2-day-old virgin males individually in clean 1-dr (15.0 × 45.0 mm) vials containing highly absorbent 1.3-cm-diameter paper disks (Schleicher and Schuell, Keene, New Hampshire) and a single cracked wheat kernel. Two hundred such vials were tightly capped, maintained at standard rearing conditions, and briefly vented at 2-day intervals to alleviate stress due to lack of oxygen. Control collections contained a disk and cracked grain only; collections from female weevils were handled in the same manner as the male collections. After 14 days, the weevils, grain, and frass were discarded. Disks were then batch extracted in 200 ml of UV-grade hexane for 24 hr. Glass wool-filtered extracts totaling 2800 insect day equivalents (IDE) were concentrated under $N_2$ to 0.5 ml, bioassayed to confirm biological activity (Phillips and Burkholder, 1981), and stored under $N_2$ at −40°C.

*Column Chromatography.* Initial purification of disk extracts was accomplished by column chromatography (LC). A 26.0 × 1.0-cm (ID) glass analytical column was packed with 60–100/PR Florisil® (Sigma Chemical Co.). The 0.5-ml volume of crude extract was then applied to the column and eluted isocratically with 1 : 1 UV-grade hexane–diethyl ether at a flow rate of 1.0 ml/min. Fifty 1.0-ml fractions of column eluent were collected and later bioassayed to determine which fractions contained pheromone. Two- to 7-day-old virgin male and

female weevils were tested against paired disks, one spotted with 5.0 $\mu$l of fraction eluent (treatment), and the other with 5.0 $\mu$l of 1 : 1 hexane–diethyl ether (control). For granary weevil bioassays, the LC fractions were concentrated (10×) under $N_2$ prior to bioassay. LC fractions were then stored under $N_2$ at −40°C.

*Preparative Gas-Liquid Chromatography (GLC).* Isolation of natural pheromone from active 1.0-ml LC fractions was accomplished by means of preparative GLC. A Varian 3700® chromatograph, equipped with a flame ionization detector (FID), was fitted with a 1.93-m × 6.35-mm (ID) stainless-steel column packed with 3% SE-30® on 80/100 mesh Gas Chrom Q® (Supelco, Inc.) (column P). A Scientific Glass Engineering (SGE)® glass-lined, stainless-steel, variable effluent splitter was installed to allow glass capillary tube collections of GLC column fractions in a dry ice trap. Biologically active 1.0-ml LC fractions were concentrated under $N_2$ to 10.0-$\mu$l volumes prior to GLC injection and subsequent pheromone collection. Column conditions during collections were: 50°C raised to 200°C at 5°C/min; injector 150°C; detector 250°C. During collections, the effluent splitter was completely open, diverting all but a small fraction of the pheromone to the collection tubes. Glass capillary collection tubes were extracted with UV-grade hexane. Bioassays were then conducted on the GLC collections to locate biological activity. These purified weevil pheromone extracts were then stored under $N_2$ at −40°C until tests on the structural features of the pheromone molecule could be conducted.

*Nuclear Magnetic Resonance (NMR) Spectroscopy.* A [$^1$H]NMR spectrum of GLC-purified male rice weevil pheromone (Schmuff et al., 1984) was recorded at 500 MHz on a Nicolet NT-500® instrument. [$^1$H]NMR spectra of synthetic materials were recorded either on the preceding instrument, or at 360 MHz on a Nicolet NT-360®, or at 220 MHz using an in-house assembled instrument equipped with an Oxford instruments® superconducting magnet. [$^{13}$C]NMR spectra of synthetic materials were recorded on a JEOL FX-60® operating at 15 MHz.

*Gas Chromatography-Mass Spectrometry (GC-MS).* GC-MS electron impact (EI-MS) analyses were carried out on purified natural pheromone, and on synthetic preparations, using either an LKB-9000® or an LKB-2091® instrument. The former was fitted with an injector maintained at 270°C and a glass column (1.8 m × 1.5 mm ID) packed with 10% SP-1000® (Supelco, Inc.) on 80/100 mesh Supelcoport® (column A). Column A was programed at 45°C for 6.4 min, then raised to 200°C at 10°C/min. The LKB-2091, utilizing direct on-column injection, was fitted with a 30-m × 0.35-mm ID fused silica capillary column wall-coated (0.25-$\mu$m film) either with SPB-1® (column B), or with Carbowax 20 M® (column C). Column B was programed at 65°C for 5.8 min, then raised to 200°C at 10°C/min. The oven temperature of column C was held isothermally at 130°C.

Chemical ionization mass spectra (CI-MS) were recorded using a Finnigan

3300-F® quadrupole instrument. The reagent gas in the positive ion mode was 0.7 torr of $CH_4$. For hydroxide ion generation, $N_2O$ was added to the normal $CH_4$ operating gas to give a gauge pressure reading of 0.9 torr.

*Gas-Liquid Chromatography.* Chromatograms on synthetic pheromone were recorded on a Shimadzu GC-mini 1® fitted with a glass column packed with 1% OV-17® on 80/100 mesh Supelcoport (column D). Column D was programed at 80°C for 4.0 min, then raised to 200°C at 10°C/min; the injector and detector temperatures were maintained at 270°C and 250°C, respectively. Preparative GC for diastereomer separation of the synthetic compounds was conducted on a Varian Aerograph 1400® fitted with a thermal conductivity detector and a stainless-steel column (1.8 m × 4.5 mm ID) packed with 10% SP-1000 on 80/100 mesh Supelcoport (column E). The oven temperature of column E was held isothermally at 150°C; both the injector and detector were maintained at 200°C.

*Racemic Pheromone Synthesis.* Method A is nearly identical to that of Smith and Levenberg (1981) for the preparation of a number of β-hydroxyketones including 5-hydroxy-4-methyl-3-heptanone itself. A solution of *N,N*-diisopropylamine (1.4 ml, 1.1 g, 10.0 mmol) was prepared in 10.0 ml of tetrahydrofuran at −78°C. To this solution was added a second solution of *n*-butyllithium in hexane (6.7 ml, 10.0 mmol). The resulting solution was immediately allowed to warm to room temperature, then recooled to −60°C. Neat 3-pentanone (1.05 ml, 861.0 mg, 10.0 mmol) was next added dropwise to the first solution. After maintaining this mixture at −60°C for 20 min, it was cooled further to −78°C, and neat propionaldehyde (794.0 μl, 639.0 μg, 11.0 mmol) was added dropwise. After 15 min at −78°C, the mixture was quenched by the addition of a few milliliters of saturated aqueous ammonium chloride. The resultant mixture was then partitioned between 20 ml of saturated ammonium chloride and 10 ml of ether. The organic layer was extracted with another 20 ml portion of ammonium chloride and evaporation of the organic layer in vacuum provided 965 mg (67%) of a crude slightly yellow oil. Back extraction of the combined aqueous phases (3 × 30 ml of ether) provided another 175 mg of product (total crude product 1.14 g, 79%).

For method B, racemic pheromone was also synthesized by the method outlined by Dubois and Luft (1954). A 1-liter, 2-necked flask equipped with a dropping funnel, thermometer, and magnetic stirrer was charged with 344.0 g (4 mol) of freshly distilled 3-pentanone. After cooling the apparatus to 15°C, an addition was made of 30.6 ml of 16.8% (w/w) potassium hydroxide in methanol. This was followed by dropwise addition over 4 hr of 58.0 g (1 mol) of freshly distilled propionaldehyde mixed with another 172.0 g lot of 3-pentanone. Stirring was continued for an additional 30 min after which the base (KOH) was neutralized with 3.5 g of anhydrous oxalic acid. The solution was filtered and then distilled under vacuum to remove unreacted 3-pentanone and methanol. The

aldol distilled at 70°C (0.45 mm) and yielded 66.7 g (46% based on propionaldehyde).

Synthesis of racemic pheromone by method A is more convenient for small preparations since no purification is required. Method B is preferred when multigram preparations are required.

To verify that the diastereomers of natural sitophilure were the same as those of the synthetic racemic pheromone, a capillary GC coinjection of both preparations was performed. An HP-5790A® (Hewlett-Packard Co.) GC was fitted with a 30-m SP-1000 (Supelco, Inc.) fused silica capillary column (column S). Column conditions were: 60°C (held 0.5 min) raised to 210°C (held 8.5 min) at 15°C/min; injector 210°C; detector 220°C. Natural pheromone totalling 46 ng was coinjected with 14 ng of the synthetic racemic material. The resulting chromatogram was then analyzed for peak heights (amounts in nanograms) and elution times that related to the coinjected materials.

*Statistical Analysis.* Bioassay data were examined by using the $t$ test for paired data. Statistical differences at the 1% level ($P < 0.01$) were accepted as biologically significant.

## RESULTS AND DISCUSSION

*Pheromone Isolation and Identification.* Bioassays indicated the presence of sitophilure in LC fractions 18–33 for both male rice and maize weevil collections. None of the granary weevil LC fractions proved to be attractive, although a response to crude extracts had been observed.

Preparative GC on active LC fractions indicated a peak at 82°C on column P which coincided with biological activity. GC collections of 2800 IDE yielded a minimum of ca. 7.5 $\mu$g of pheromone from both the rice and maize weevil collections. Comparisons of preparative GC peak areas indicated that maize weevil pheromone production was at least four times greater than that of the rice weevil. A biologically active substance with the above GC retention characteristics was not detectable for any of the concentrated grain control, female rice or maize weevil, or granary weevil (both sexes) LC fractions. In addition, capillary GC analyses showed only small peaks at retention times characteristic of the natural pheromone in a 7100 IDE male granary weevil sample. These peaks suggested the presence of less than 6.0 ng of pheromone per concentrated granary weevil LC fraction in fractions that corresponded to biologically active rice and maize weevil LC fractions. In contrast, active rice and maize weevil LC fractions often contained microgram quantities of the pheromone.

Table 1 shows the retention times and temperatures (when temperature programed) of natural and synthetic pheromones on GC columns A–S used during the course of this work. On all analytical systems, natural and synthetic pher-

TABLE 1. GLC CHARACTERISTICS OF NATURAL AND SYNTHETIC SITOPHILURE

| Column | Elution time (min) | Elution temp. |
|---|---|---|
| A | 19.2 | 161°C (10°C/min) |
| B | 10.3 | 95°C (10°C/min) |
| C | 5.1 (*R**,*R**) | 130°C (isothermal) |
| | 5.4 (*R**,*S**) | same |
| D | 6.2 | 108°C (10°C/min) |
| E | 9.8 (*R**,*R**) | 150°C (isothermal) |
| | 11.2 (*R**,*S**) | same |
| P | 6.4 | 82°C (5°C/min) |
| S | 11.02 (*R**,*R**) | 210°C (15°C/min) |
| | 11.19 (*R**,*S**) | same |

omones showed the same retention times. The $R^*,S^*$ and $R^*,R^*$ diastereomers are easily distinguished on polar packed columns or on nonpolar capillary columns. The $R^*,S^*$ diastereomer invariably eluted later, presumably due to more steric hindrance of its intramolecular hydrogen bonding. Elucidation of the structure of the pheromone relied heavily on its mass spectrum, presented in part in Schmuff et al. (1984). Figure 1 presents complete mass spectra of both synthetic diastereomers. The relative intensities of $M^{+\cdot} - Et$ and $M^{+\cdot} - H_2O$ are small but reproducible and allow the diastereomers to be reliably differentiated on this basis. The spectrum of the natural pheromone was virtually identical to that of the synthetic $R^*,S^*$ diastereomer. High-resolution mass measurements were performed on several ions of the natural product: $m/z$ 126.1037 ($C_8H_{14}O$); 115.0767 ($C_6H_{11}O_2$); 97.0658 ($C_6H_9O$); 86.0716 ($C_5H_{10}O$); 70.0057 ($C_3H_2O_2$).

The negative ion Cl-MS of the natural pheromone ($CH_4$, $N_2O$) showed important ions at $m/z$ 176 [7, $(M + O_2)$]; 143 [13, $(M - H)^-$]; 125 [11, $(M - H - H_2O)$]; 85 (100). The last peak corresponds to the enolate anion of 3-pentanone, presumably derived from retroaldol cleavage. This ion would not be expected from related 1,2- or 1,4-hydroxyketone systems.

The positive ion Cl-MS ($CH_4$) of the natural pheromone exhibited intense ions at $m/z$ 145 [28, $(M + H)^+$]; 127 (73, $[M + H - H_2O)^+$]; 115 (32); 99 (18); 87 (100); 75 (25). The retroaldol cleavage, now "acid" catalyzed, is obvious in the abundant protonated 3-pentanone ion at $m/z$ 87.

The [$^1$H]NMR spectrum of the natural product was complicated by the presence of contaminants, but as discussed earlier (Schmuff et al., 1984) certain structural features were discernible. Synthesis and purification (>99%) by GC (column E) provided samples of the two isomers for complete analysis and subsequent bioassays: (1) [$^1$H]NMR (500 MHz, $CDCl_3$) for the $R^*,S^*$ diastereomer: 0.97 (3H, t, $J$ = 7.4 Hz); 1.05 (3H, 5, $J$ = 7.2 Hz); 1.17 (3H, d, $J$ = 7.1 Hz);

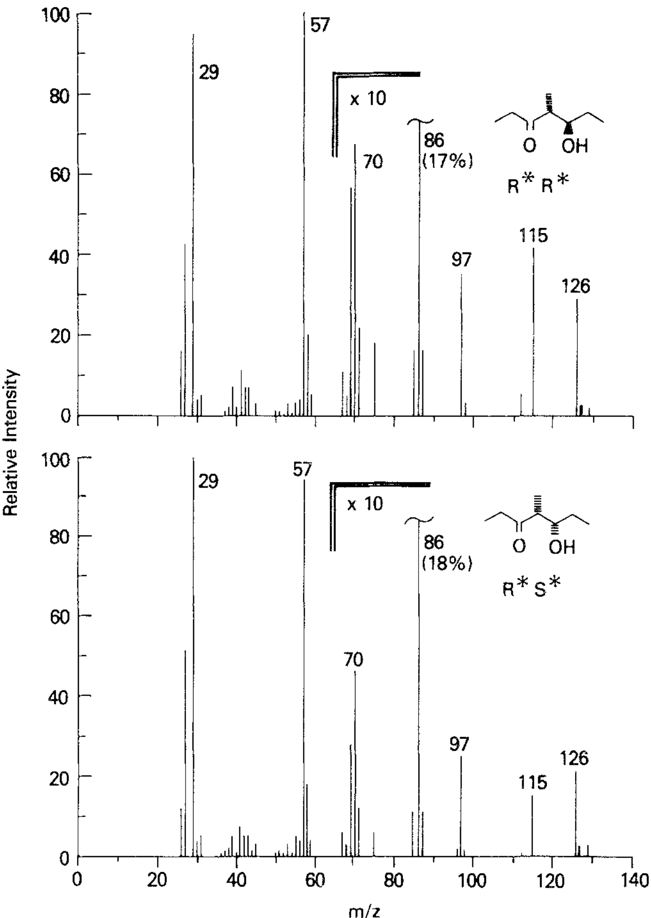


FIG. 1. Complete mass spectra (EI-MS) of synthetic (*R**,*R**)- and (*R**,*S**)-5-hydroxy-4-methyl-3-heptanone, utilizing a wall-coated, open tubular (WCOT) Carbowax 20 m capillary GLC column (column C) interfaced with a LKB-2091® mass spectrometer. The column was held isothermally at 130°C.

1.41 (1H, m); 1.56 (1H, m); 2.46 (1H, m); 2.57 (1H); 2.66 (1H, m); 3.61 (1H, m). (2) [$^{1}$H]NMR (500 MHz, $CDCl_3$) for the *R**,*R** diastereomer: 0.95 (3H, t, $J$ = 7.4 Hz); 1.05 (3H, t, $J$ = 7.2 Hz); 1.12 (3H, d, $J$ = 7.1 Hz); 1.38 (1H, m); 1.52 (1H, m); 2.50 (1H, m); 2.55 (1H, m); 2.60 (1H, m); 3.81 (1H, m).

The identity of these two diastereomers as *R**,*S** and *R**,*R** was confirmed by comparison of the [$^{13}$C]NMR spectrum of our synthetic isomeric mixture with the data reported by Heathcock et al. (1979), who have previously assigned the stereochemistry of the 5-hydroxy-4-methyl-3-heptanones. These data support the *R**,*S** diastereomer as the major component of the naturally produced

aggregation pheromone of the rice and maize weevils. Additional GC analyses indicated that there is less than 0.5% of the *R**,*R** diastereomer relative to the *R**,*S** in the natural, freshly extracted rice and maize weevil male aggregation pheromone. It is noteworthy that upon further manipulation (column chromatography, standing in $CHCl_3$, etc.), the proportion of the *R**,*R** diastereomer was substantially increased. Capillary GC analyses indicated that sitophilure production by male rice weevils was ca. 3–6 ng/day (1 IDE) in comparison to ca. 25–30 ng/day by male maize weevils.

Synthesis of racemic diastereomers, prepared either by lithium enolate condensation (method A), or the more classical sodium hydroxide procedure (method B), proceeded smoothly. By the first procedure, the crude product was obtained in >90% purity ([$^1$H]NMR, GC) as a 2:1 mixture of the *R**,*S** and *R**,*R** diastereomers. Method B gave a product that required distillation but yielded the two diastereomers in nearly equal amounts.

A capillary GC coinjection of the natural maize weevil pheromone with the synthetic racemic diastereomers (from procedure B) produced a chromatogram with two peaks only (Figure 2). The elution times (11.02 and 11.19 min) of the two peaks were identical to those previously determined for (*R**,*R**)- and (*R**,*S**)-5-hydroxy-4-methyl-3-heptanone standards. The detected amount of the *R**,*R** diastereomer was 10 ng, 7 ng from the synthetic material plus 3 ng from the natural pheromone. The *R**,*S** diastereomer totaled 50 ng, 7 ng from the synthetic material plus 43 ng from the natural pheromone. Thus, these criteria indicate that the natural and synthetic diastereomers are identical.

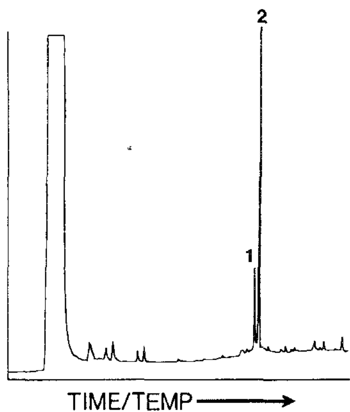


FIG. 2. Coinjection of naturally produced sitophilure with the synthetic racemic pheromone. Column S (see text and Table 1): SP-1000 (Supelco, Inc.) 30-m capillary. Peak 1 coincides exactly with the elution time (11.02 min) of (*R**,*R**)-5-hydroxy-4-methyl-3-heptanone, and peak 2 appears at 11.19 min, the elution time of the *R**,*S** diastereomer. Peaks 1 and 2 represent 10 and 50 ng, respectively, the total amounts coinjected of the natural and synthetic diastereomers.

*Attractiveness of Synthetic Pheromone.* Both sexes of rice and maize weevils showed highly significant ($P < 0.001$) responses to either the $R^*,S^*$ or $R^*,R^*$ diastereomer of synthetic sitophilure and to a 1 : 1 mixture of the two (Figure 3). Rice weevils, however, have a lower optimal response dosage (30 ng) than maize weevils (100–400 ng). Even though production of sitophilure by male granary weevils is in doubt, females responded strongly ($P < 0.01$) to doses from 200–800 ng of the $R^*,S^*$ diastereomer of the synthetic pheromone. Response by males was inconsistent and generally weaker than that by females. The synthetic $R^*,R^*$ diastereomer was somewhat attractive ($P < 0.05$) to both sexes of the granary weevil, but this overall response was weaker than that elicited by the $R^*,S^*$ diastereomer. Combining the $R^*,R^*$ and $R^*,S^*$ diastereomers did not improve the response of granary weevils over those observed for each individual component.

Although no other compound with the sitophilure substructure has been reported as an insect pheromone (as judged from a substructure search of *Chemical Abstracts* and *Index Chemicus* using the DARC/Questel® system), it fits the polyketide biosynthetic pattern seen in different insect orders (Chuman, 1981;

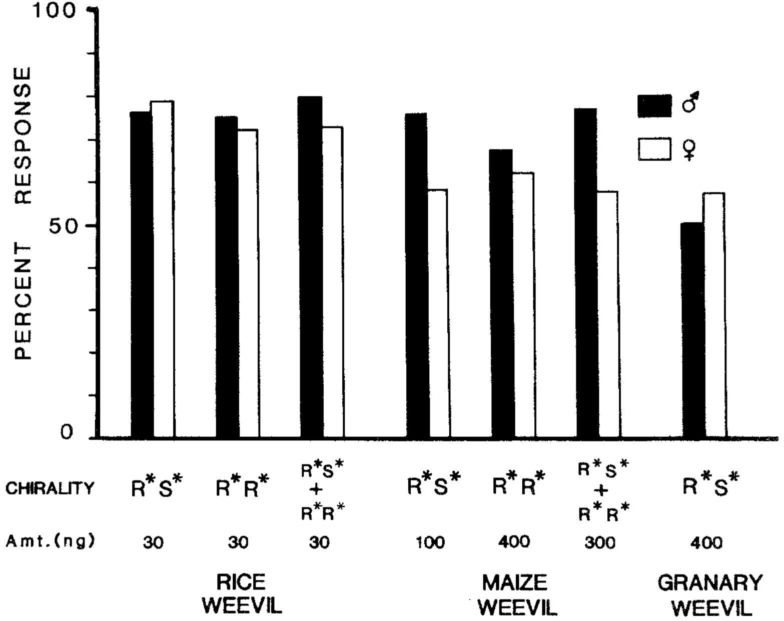


FIG. 3. Attractiveness of the $R^*,R^*$ and $R^*,S^*$ diastereomers of synthetic, racemic sitophilure, singly or in combination (1 : 1), to males and females of the rice and maize weevils, and of the $R^*,S^*$ diastereomer to both sexes of the granary weevil. Amounts of pheromone in nanograms represent dosages for optimal response when tested in a dual-choice pitfall bioassay system. Responses are based on the average of 10 repetitions, each with 10 virgin insects.

FIG. 4. Some propionate-derived compounds and presumed precursors utilized for chemical communication or defense: (a) Sitophilure, a male-produced aggregation pheromone of *Sitophilus oryzae* (L.) and *S. zeamais* Motsch.; absolute stereochemistry unknown; (b) a male-produced aggregation pheromone of *Sitona lineatus* (*L.*) the pea and bean weevil (Blight et al., 1984); (c) a volatile component from "daddy longlegs" (Arachnida: Opiliones: *Leiobunum* spp.) defense glands (Jones et al., 1977); (d) two compounds occurring in the volatiles produced by elm bark beetles (*Scolytus* spp.) invading host elm trees (Blight et al., 1979, 1983); (e) the two forms of serricornin, the sex pheromone of the female cigarette beetle (*Lasioderma serricorne* F.) (Mori et al., 1984); (f) serricorone, a sex pheromone isolated from the female cigarette beetle (*L. serricorne*) (Chuman et al., 1983) (only the relative *cis* configuration of the indicated 2,3-alkyl groups is known); (g) multistriatin, an aggregation pheromone produced by virgin female European bark beetles (*S. multistriatus*) as they tunnel in host elms (Plaumann et al., 1982; Mori, 1976; Pearce et al., 1976).

Francke et al., 1982) with natural sitophilure being the product of the propionate pathway. Sitophilure is the first coleopteran product to exhibit the 1,3 oxygenation pattern expected for polyketides, although this pattern can be inferred in several arthropod-derived structures that display uncyclized and/or hydrated forms (Figure 4).

3-Pentanone was found in small quantities in volatiles from male rice and maize weevils, and may be a degradation product of 5-hydroxy-4-methyl-3-heptanone. It has not been detected in volatiles from females or wheat. The role of 3-pentanone as part of the pheromone complex is still in question. However, it does appear, at least in young weevils, to improve pheromone response. Young

males and females show some attraction to 3-pentanone by itself, and a combination of equal parts of 3-pentanone and the synthetic racemic pheromone produced higher responses than to either compound alone. Aged weevils responded best to 3-pentanone when starved, but since it has not been found in wheat volatiles, its function as a feeding attractant is in doubt (Walgenbach and Burkholder, unpublished data).

The results of this study confirm previous data on interspecific attraction of *Sitophilus* spp. weevils (Walgenbach et al., 1983). The maize and rice weevils appear to be more closely related to each other than to the granary weevil. Thus rice and maize weevils produce sitophilure while rice, maize, and granary weevils all respond to this pheromone. Studies on the attractant produced by the granary weevil, and on the potential for use of sitophilure to manage weevil infestations, are in progress.

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# STUDIES ON RELATIONSHIP BETWEEN ACTIVITY AND ELECTRON DENSITY ON CARBONYL OXYGEN IN SEX PHEROMONE MIMICS OF THE AMERICAN COCKROACH, PART XI[1]

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**Abstract**—From the relationship between structures of the 2$\alpha$-substituents of verbanyl analogs and their sex pheromone activities to the American cockroach, electron density of the carbonyl oxygen atom in the substituent, as estimated by the [$^{17}$O]NMR chemical shift, was estimated to be an important factor which influenced the activity, in addition to length of the substituent and the position of the carbonyl group. (+)-Verbanyl methylcarbonate (XX), possessing the highest electron density on the carbonyl oxygen atom, showed the strongest activity among the analogs.

**Key Words**—Sex pheromone mimics, American cockroach, *Periplaneta americana*, Orthoptera, Blattidae, (+)-Verbanyl methylcarbonate, electron density, [$^{17}$O]NMR.

## INTRODUCTION

Since we found (+)-*trans*-verbenyl acetate (I in Figure 1) to be a sex pheromone mimic of the American cockroach, *Periplaneta americana* L. (Nishino et al., 1977), structural factors for sex pheromone activity implicit in I have been investigated (Nishino and Takayanagi, 1981; Takayanagi and Nishino, 1982; Nishino et al., 1982; Manabe et al., 1983). The carbonyl oxygen atom of the

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α-oriented C-2 ester group of I was assumed to play the most important role for inducing pheromonal activity as an electron donor to the corresponding receptor site (Nishino and Takayanagi, 1981). In addition, the position of the carbonyl group in the ester group with an appropriate length was also suggested to be an important factor (Nishino and Takayanagi, 1981; Nishino et al., 1982). Apart from the ester group, the existence and length of the C-4 and C-6 methyl groups of I were revealed as the important factors (Nishino and Takayanagi, 1981; Takayanagi and Nishino, 1982; Manabe et al., 1983).

In the above structure–activity studies, (+)-verbanyl analogs were found to have stronger activities than the corresponding (+)-*trans*-verbenyl analogs (for example, see activities of I and XVII in Table 1). Therefore, (+)-verbanyl analogs were systemically synthesized in this work, considering the position of the carbonyl group and the length of the C-2 substituent (Figures 1 and 2), and tested for sex pheromone activity to male cockroaches. Based on the results of the structure–activity relationship, (+)-verbanyl methylcarbonate (XX) with more potent activity was prepared. This paper also deals with the possibility of using the $^{17}O$ chemical shift value of the carbonyl oxygen atom in [$^{17}O$]NMR as an indicator of the electron density.

## METHODS AND MATERIALS

*Instrumentation.* Specific rotations were measured in *n*-hexane at 25°C with a Union automatic polarimeter PM-201. Infrared (IR) spectra were recorded on a JASCO IRA-1. [$^{1}H$]NMR spectra were taken in $CDCl_3$ and TMS (internal standard) on a JEOL FX-90Q (90 MHz) or a Hitachi R-24 (60 MHz). Chemical shifts (ppm) were measured from the TMS signal. Gas chromatography (GC) was performed with a Shimadzu 4BM-PF using a 2-m × 3-mm column packed with 3% OV-225 at 110°C, and $N_2$ gas (40 ml/min) as carrier gas. Mass spectra (MS) were measured at 70 eV with a Shimadzu GCMS-7000 in which the column used was the same as in GC analysis.

*Synthesis.* Analogs III–XIII and analogs XV–XXI were synthesized, respectively from (+)-verbanone (II) and (+)-verbanol (XIV) which were previously obtained (Nishino and Takayanagi, 1979) (Figure 1). Esters XVII (Nishino and Takayanagi, 1981) and XVIII (Nishino et al., 1984) were previously synthesized. Dried solvents were used for all reactions. Analogs were purified by silica gel column chromatography eluting with mixed solvents of *n*-hexane (H) with ethyl acetate (EtOAc) unless otherwise stated. Purity of the analogs was evaluated to be almost 100% by GC.

*[1R-(1α,2α,4α,5α)]-4,6,6-Trimethylbicyclo[3.1.1]heptane-2-carbaldehyde (III).* To a mixture of methoxymethyltriphenylphosphonium chloride (1.37 g) with benzene (50 ml) and tetrahydrofuran (THF) (50 ml), 1.6 N *n*-hexane solution of *n*-butyllithium (24.8 ml) was added dropwise at −10°C in an argon

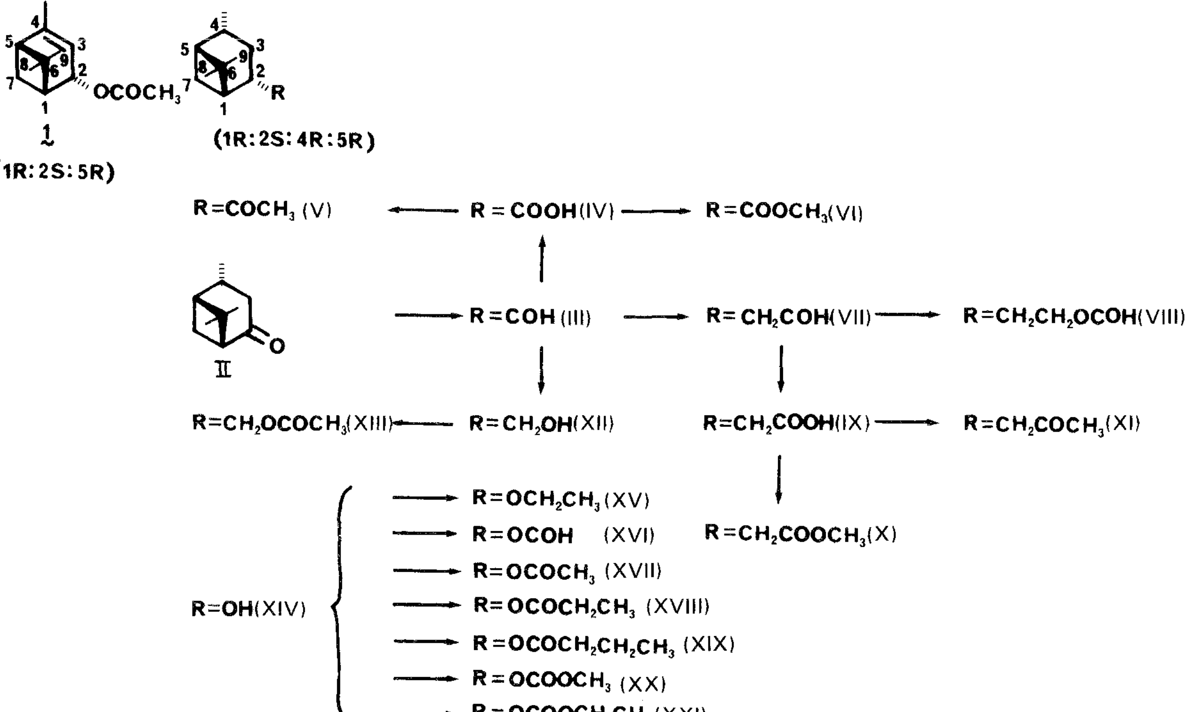


FIG. 1. Synthetic routes to verbanyl analogs with 2-substituents. In compounds VII–XIII, the absolute configuration of C-2 should be 2*R*.

gas atmosphere and stirred for 30 min. A solution of II (1.5 g) in benzene was added dropwise to the reaction mixture, and stirred at room temperature overnight. The reaction mixture was poured into ice-water and extracted with *n*-pentane. The pentane-soluble fraction was chromatographed (*n*-pentane) to separate an oily material from triphenylphosphonium oxide. After the oily material was treated with 10% aqueous HCl (3 ml) and THF (3 ml) at room temperature for 2.5 hr, water (10 ml) was added, and it was extracted with *n*-pentane. The concentrate of the *n*-pentane solution was chromatographed eluting with H, H/EtOAc = 3:1 and 25:1, succesively, to give III (400 mg), $[\alpha]_D = +18.4°$ (*c* 1.00); IR (film): 2805, 2700, 1723 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.83 (3H, s) 0.87 (3H, d, $J$ = 6.0 Hz), 1.21 (3H, s), 2.59 (1H, broad t, $J$ = 9.0 Hz), 9.46 (1H, d, $J$ = 1.3 Hz); MS: *m/e* 166 ($M^+$, $C_{11}H_{18}O$), 151 ($M^+ - CH_3$), 148, 137 ($M^+$ − CHO), 136 ($M^+$ − HCHO), 121, 107, 93 [base peak ($B^+$)], 85, 83, 81; retention time ($t_R$) in GC: 9.1 min.

*(+)-Verbanylcarboxylic Acid (IV) and Its Methyl Ester (VI).* Jones oxidation ($CrO_3$–$H_2SO_4$ in water–acetone) of III (200 mg) gave a crude crystal of IV which was recrystallized from *n*-hexane to give pure IV (170 mg), mp 68.5–69.5°C; $[\alpha]_D = +14.0°$ (*c* 0.50); IR (KBr): 2880, 1690, 925 $cm^{-1}$; [$^1$H]NMR (60 MHz); 0.81 (3H, s), 0.87 (3H, d, $J$ = 6.0 Hz), 1.21 (3H, s), 2.83 (1H, broad t, $J$ = 8.5 Hz), 11.30 (1H, broad s); MS: *m/e* 182 ($M^+$, $C_{11}H_{18}O_2$), 167 ($M^+ - CH_3$), 164, 149 ($M^+ - CH_3OH$), 139, 137 ($M^+$ − COOH), 126, 122, 121, 114 ($B^+$), 97, 95, 93, 86, 83, 81. Acid IV was esterified with diazomethane in ether to afford methyl ester VI, $[\alpha]_D = +15.9°$ (*c* 1.00); IR (film): 1735, 1195, 1170 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.80 (3H, s), 0.86 (3H, d, $J$ = 6.0 Hz), 1.18 (3H, s), 2.78 (1H, broad t, $J$ = 8.5 Hz), 3.60 (3H, s); MS: *m/e* 196 ($M^+$, $C_{12}H_{20}O_2$), 185 ($M^+ - CH_3$), 164, 153, 149, 137 ($M^+ - CO_2CH_3$), 136 ($M^+$ − $HCO_2CH_3$), 128 ($B^+$), 122, 121 ($B^+$), 97, 95, 93, 83, 81; $t_R$: 12.9 min.

*[1R-(1α,2α,4α,5α)]-4,6,6-Trimethylbicyclo[3.1.1]heptane-2-carbonylmethane (V).* To IV (70 mg) in benzene (1.34 ml), oxalyl chloride (0.23 ml) was added under cooling with ice-bath, stirred for 1.5 hr, and concentrated to give the acid chloride. The acid chloride was dissolved into ether (0.8 ml) and added dropwise to an ether solution of lithium dimethylcuprate (3.8 ml), which was prepared from cuprous iodide (220 mg) and 1.55 N hexane solution of methyllithium (1.48 ml), at −78°C for 10 min. After methanol (1 ml) was added to the reaction mixture and stirred for 5 min, water (30 ml) was added, and the mixture was extracted with ether. A concentrate of the ether solution was chromatographed (H/EtOAc = 15 : 1) to give V (53 mg), $[\alpha]_D = +37.0°$ (*c* 1.00); IR (film): 1710 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.85 (3H, s), 0.90 (3H, d, $J$ = 6.0 Hz), 1.24 (3H, s), 2.09 (3H, s), 2.88 (1H, broad t, $J$ = 9.0 Hz); MS: *m/e* 180 ($M^+$, $C_{12}H_{20}O$), 165 ($M^+ - CH_3$), 137 ($B^+$, $M^+ - COCH_3$), 121, 113, 97, 95, 83, 81; $t_R$: 15.2 min.

*[1R-(1α,2σ,4α,5α)]-4,6,6-Trimethylbicyclo[3.1.1]heptane-2-acetaldehyde (VII).* This aldehyde (VII) (500 mg) was obtained from III (900 mg) by a Wittig

reaction and subsequent acid hydrolysis as performed for obtaining III from II. VII, $[\alpha]_D$ = +0.7° (*c* 1.00); IR (film): 2810, 2705, 1725 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.79 (3H, d, *J* = 6.0 Hz), 0.81 (3H, s), 1.17 (3H, s), 9.73 (1H, t, *J* = 2.5 Hz); MS: *m*/*e* 180 ($M^+$, $C_{12}H_{20}O$), 165 ($M^+$ − $CH_3$), 162, 147, 137 ($M^+$ − $CH_2CHO$), 136 ($M^+$ − $CH_3CHO$), 119, 111, 109, 107, 93, 83 ($B^+$), 81; $t_R$: 16.3 min.

*Formate (VIII) of (+)-Verbanyl Ethanol.* Lithium alminium hydride (excess) reduction of VII (50 mg) (in ether at −15°C for 2 hr) afforded crude (+)-verbanyl ethanol which was purified by chromatography (H/EtOAc = 10:1) to give the ethanol (59 mg). The ethanol was treated with 85% HCOOH aqueous solution at 60°C for 3 hr. From the reaction mixture, VIII was purified by chromatography (H/EtOAc = 10:1). VIII, $[\alpha]_D$ = −1.4° (*c* 1.00); IR (film): 1725, 1170 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.80 (3H, s), 0.83 (3H, d, *J* = 6.0 Hz), 1.19 (3H, s), 1.63 (2H, double t, *J* = 7.0, 7.0 Hz), 4.16 (2H, t, *J* = 7.0 Hz), 8.04 (1H, s); MS: *m*/*e* 210 ($M^+$, $C_{13}H_{22}O_2$), 195 ($M^+$ − $CH_3$), 181 ($M^+$ − CHO), 167, 164 ($M^+$ − $HCO_2H$), 153, 149, 141, 137 ($M^+$ − $CH_2CH_2COH$), 136 ($M^+$ − $CH_3CH_2OCOH$), 121, 107, 95, 93, 83 ($B^+$), 81; $t_R$: 27.8 min.

*[1R-(1α,2α,4α,5α)]-4,6,6-Trimethylbicyclo[3.1.1]heptane-2-acetic Acid (IX) and its Methyl Ester (X).* Jones oxidation ($CrO_3$–$H_2SO_4$ in water–acetone) of VII (250 mg) and subsequent chromatographic purification (H/EtOAc = 1:2) yielded IX as colorless oil, $[\alpha]_D$ = +2.8° (*c* 1.00); IR (film): 3080, 2660, 1705, 930 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.80 (3H, d, *J* = 6.0 Hz), 0.81 (3H, s), 1.18 (3H, s), 10.99 (1H, broad s); MS: *m*/*e* 196 ($M^+$, $C_{12}H_{20}O_2$), 181 ($M^+$ − $CH_3$), 178 ($M^+$ − $H_2O$), 167, 163, 153, 140, 136 ($M^+$ − $CH_3CO_2H$), 127, 121, 107, 100, 95, 93, 85, 83 ($B^+$), 81. On treatment with diazomethane, IX gave crude X which was purified by chromatography (H/EtOAc = 20:1) to give pure X, $[\alpha]_D$ = −1.7° (*c* 1.00); IR (film): 1730, 1160 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.78 (3H, d, *J* = 6.0 Hz), 0.80 (3H, s), 1.15 (3H, s), 3.59 (3H, s); MS: *m*/*e* 210 ($M^+$, $C_{13}H_{22}O_2$), 195 ($M^+$ − $CH_3$), 181, 179 ($M^+$ − $OCH_3$), 178, 167, 163, 161, 154, 150 ($M^+$ − $HCO_2CH_3$), 141, 136 ($B^+$), 121, 109, 107, 99, 95, 83, 81; $t_R$: 20.2 min.

*[1R-(1α,2α,4α,5α)]-4,6,6-Trimethylbicyclo[3.1.1]heptane-2-acetone (XI).* From IX (100 mg), crude XI was obtained on similar treatment with oxalyl chloride and lithium dimethylcuprate as in the case of the formation of V from IV. The crude XI was purified by chromatography (H/EtOAc = 15:1). XI (80 mg), $[\alpha]_D$ = +1.0° (*c* 1.00); IR: 1715 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.82 (3H, d, *J* = 6.0 Hz), 0.85 (3H, s), 1.19 (3H, s), 2.08 (3H, s); MS: *m*/*e* 194 ($M^+$, $C_{13}H_{22}O$), 179 ($M^+$ − $CH_3$), 176 ($M^+$ − $H_2O$), 161, 151 ($M^+$ − $COCH_3$), 136 ($B^+$), 125, 121, 113, 109, 107, 97, 95, 83, 81; $t_R$: 22.8 min.

*[1R-(1α,2α,4α,5α)]-4,6,6-Trimethylbicyclo[3.1.1]heptane-2-methanol (XII) and its Acetate (XIII).* From III (60 mg), XII (59 mg) was prepared by lithium alminium hybride reduction and chromatographic purification. XII, $[\alpha]_D$ = −12.7° (*c* 1.00); IR (film): 3320 $cm^{-1}$; [$^1$H]NMR (90 MHz): 0.68 (1H, doublet,

$J$ = 13.6, 8.9 Hz), 0.84 (3H, s), 0.86 (3H, d, $J$ = 6.6 Hz), 1.24 (3H, s), 3.41 (2H, d, $J$ = 6.6 Hz); MS: *m/e* 168 ($M^+$, $C_{11}H_{20}O$), 153 ($M^+ - CH_3$), 150 ($M^+ - CH_3$), 150 ($M^+ - H_2O$), 137 ($M^+ - CH_2OH$), 135, 125, 121, 107, 95, 83, 81 ($B^+$); $t_R$: 18.1 min. The methanol (XII) was acetylated with acetic anhydride in pyridine to give XIII, $[\alpha]_D = -9.8°$ (*c* 1.00); IR (film): 1740, 1245 $cm^{-1}$; [$^1$H]NMR (90 MHz): 0.84 (3H, s), 0.86 (3H, d, $J$ = 6.4 Hz), 1.23 (3H, s), 2.04 (3H, s), 3.84 (2H, d, $J$ = 7.0 Hz); MS: *m/e* 210 ($M^+$, $C_{13}H_{22}O_2$), 168 ($M^+ - H_2C{=}C{=}O$), 167 ($M^+ - CH_3CO$), 150 ($M^+ - CH_3CO_2H$), 135, 121, 107 ($B^+$), 95, 93, 83, 82; $t_R$: 20.7 min.

*Ethyl Ether (XV) of (+)-Verbanol (XIV).* To a suspension of sodium hydride (75 mg) in dimethyl sulfoxide (3 ml), a solution of XIV (150 mg) in ether (1 ml) and ethyl iodide (780 mg) was added successively and stirred at room temperature overnight under argon gas atmosphere. A saturated aqueous solution of $NH_4Cl$ was added to the reaction mixture under cooling with an ice-bath and extracted with ether. The ether soluble fraction was chromatographed (H/EtOAc = 20 : 1) to give XV (155 mg), $[\alpha]_D = -2.3°$ (*c* 1.00); IR (film): 1115, 1080, 1013 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.76 (3H, s), 0.87 (3H, d, $J$ = 6.0 Hz), 1.12 (3H, t, $J$ = 7.0 Hz), 1.20 (3H, s), 3.40 (2H, q, $J$ = 7.0 Hz), 3.65 (1H, broad t, $J$ = 7.5 Hz); MS: *m/e* 182 ($M^+$, $C_{12}H_{22}O$), 167 ($M^+ - CH_3$), 153 ($M^+ - CH_2CH_3$), 138, 136, 125, 121, 113 ($B^+$), 110, 99, 97, 95, 93, 85, 81; $t_R$: 3.9 min.

*(+)-Verbanyl Formate (XVI).* The esterification procedure for XIV followed that used in obtaining VIII from VII. XVI, $[\alpha]_D = +14.6°$ (*c* 0.70); IR (film): 1725, 1180 $cm^{-1}$; [$^1$H]NMR (90 MHz): 0.87 (3H, s), 0.93 (3H, d, $J$ = 6.4 Hz), 1.26 (3H, s), 5.25 (1H, broad t, $J$ = 7.5 Hz), 7.99 (1H, s); MS: *m/e* 182 ($M^+$, $C_{11}H_{18}O_2$), 167 ($M^+ - CH_3$), 153 ($M^+ - CHO$), 136 ($M^+ - HCO_2H$), 127, 126, 121 ($M^+ - CH_3 - HCO_2H$), 113, 107, 94 ($B^+$), 85, 81; $t_R$: 10.4 min.

*(+)-Verbanyl Butyrate (XIX).* The butyrate (XIX) was obtained from XIV with butyric anhydride and pyridine. XIX, $[\alpha]_D = +12.6°$ (*c* 0.50); IR (film): 1730, 1180 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.86 (3H, s), 0.92 (3H, d, $J$ = 6.0 Hz), 0.95 (3H, t, $J$ = 7.0 Hz), 2.17 (2H, q, $J$ = 7.0 Hz), 5.15 (3H, broad t, $J$ = 7.5 Hz); MS: *m/e* 224 ($M^+$, $C_{14}H_{24}O_2$), 209 ($M^+ - CH_3$), 181 ($M^+ - CH_2CH_2CH_3$), 169, 155, 136 ($M^+ - C_3H_7CO_2H$), 121 ($B^+$), 107, 93, 85, 81; $t_R$: 25.2 min.

*(+)-Verbanyl Methylcarbonate (XX) and (+)-Verbanyl Ethylcarbonate (XXI).* To a solution of XIV (50 mg) in pyridine (0.1 ml), methyl chloroformate (0.025 ml) was added, stirred for 10 min in an ice bath, and further stirred at 50°C for 3 hr. After the reaction mixture was cooled to room temperature, ice and water (5 ml) was added, and the mixture was extracted with ether. The ether soluble material was chromatographed (H/EtOAc = 20 : 1) to give XX (49.6 mg), $[\alpha]_D = +8.3°$ (*c* 1.00); IR (film): 1745, 1270 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.86 (3H, s) 0.93 (3H, d, $J$ = 6.0 Hz), 1.26 (3H, s), 3.76 (3H, s), 5.03

(1H, broad t, $J$ = 7.5 Hz); MS: $m/e$ 212 ($M^+$, $C_{12}H_{20}O_3$), 197 ($M^+$ − $CH_3$), 157, 156, 153 ($M^+$ − $CO_2CH_3$), 143, 136 ($M^+$ − $CH_3OCO_2H$), 121 ($B^+$), 107, 99, 93, 81; $t_R$: 25.7 min. Similarly, XXI was prepared from XIV with ethyl chloroformate. XXI, $[\alpha]_D$ = +5.7° ($c$ 1.00); IR (film): 1743, 1265 $cm^{-1}$; [$^1$H]NMR (60 MHz): 0.83 (3H, s), 0.89 (3H, d, $J$ = 6.0 Hz), 1.21 (3H, s), 1.24 (3H, t, $J$ = 7.0 Hz), 4.12 (2H, q, $J$ = 7.0 Hz), 4.98 (1H, broad t, $J$ = 7.5 Hz); MS: $m/e$ 226 ($M^+$, $C_{13}H_{22}O_3$), 211 ($M^+$ − $CH_3$), 171, 170, 167 ($M^+$ − $CO_2C_2H_5$), 157, 136 ($M^+$ − $C_2H_5OCO_2H$), 121 ($B^+$), 107, 93, 85, 83; $t_R$: 40.0 min.

*[$^{17}$O]NMR Measurement and Isopropyl Analogs.* Spectra of neat liquid (0.5 ml) samples in a spherical cell in a 10-mm tube were taken with a JEOL FX-90Q at 12.11 MHz (24°C). Dioxane was used as external standard. Pulse width was 20 sec (90° pulse), and the number of pulses was 3000–13000. Isopropyl methylcarbonate (bp 120°C) was prepared from 2-propanol and methyl chloroformate in pyridine [0°C to room temperature (1.5 hr) to 60°C (1 hr)]. The other analogs were obtained commercially (Tokyo Kasei Co.) and used after distillation.

*Behavioral Assay.* The assay method followed Nishino et al. (1980). A stock solution of a testing compound was made by dissolving 10 mg of the compound in 1 ml of $n$-hexane. Then 50, 10, 5, 2, 0.5, 0.25, and 0.05 $\mu$l of the solution, corresponding to 500, 100, 50, 20, 5, 2.5 and 0.5 $\mu$g of the compound, were used in tests. The dose of 500 $\mu$g was first tested. When typical sexual display (wing vibration, extended abdomen, and attempted copulation) was observed at the 500-$\mu$g level, testing quantity was reduced in the above order.

The assay was performed in a special controlled-environment assay room (26°C, 40% relative humidity, and 7:17 light–dark photoperiod) using a testing cage (24 × 30 × 9 cm) housing 25 males of the American cockroach, which had been isolated from females for at least one month. The assay was initiated at 9:00 PM under dim light. The number of males displaying the typical sexual behavior was counted within 3 min.

## RESULTS AND DISCUSSION

Analogs, except for carbonates XX and XXI, were first synthesized and tested for sex pheromonal activity with males of the American cockroach. However, all the analogs and intermediates except for X, XIII, and XVI–XVIII were inactive at less than 500 $\mu$g. The length of C-2 substituents and the position of the carbonyl groups in the synthesized analogs are demonstrated in Figure 2. The assay results are listed in Table 1.

Besides (+)-verbanol (XIV), the ethyl ether XV was inactive, despite having the same arrangement of atoms (—O—C—C) in the substituent as in that of pheromonally active ester XVII. This implies no significant function of the alcoholic oxygen atom for inducing pheromone activity. Esterification of XIV

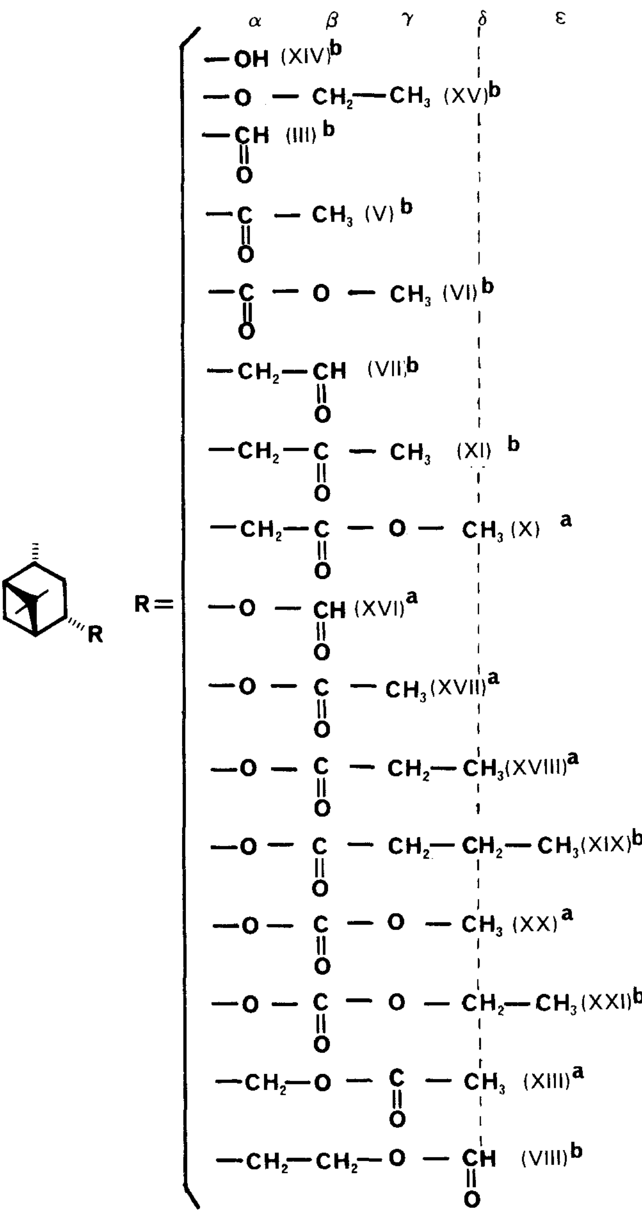


FIG. 2. Length and position of carbonyl group of C-2 substituents in verbanyl analogs. (a) Sex pheromonally active analogs; (b) Inactive analogs. For inducing pheromone activity, β-carbonyl group is required as the most suitable position in ester groups whose length is limited within the broken line (δ-position).

TABLE 1. SEX PHEROMONE ACTIVITY OF VERBANYL ANALOGS TO MALES OF AMERICAN COCKROACH AND $^{17}O$ CHEMICAL SHIFTS OF CARBONYL OXYGEN ATOMS OF ISOPROPYL ANALOGS CORRESPONDING TO SEVERAL VERBANYL ANALOGS

| Verbanyl analog (see Figure 2) | Quantity ($\mu$g) | Number of repetitions | Activity[a] | $\delta(^{17}O)$ (C=O) (ppm) |
|---|---|---|---|---|
| | | | | $(CH_3)_2$ CH-R |
| XVI | 500 | 5 | NA[b] | NM[c] |
| XV | 500 | 5 | NA | NM |
| III | 500 | 5 | NA | NM |
| V | 500 | 5 | NA | NM |
| VI | 500 | 5 | NA | NM |
| VII | 500 | 5 | NA | NM |
| XI | 500 | 5 | NA | 571 |
| X | 100 | 10 | 5 ± 4 | 364 |
| XVI | 100 | 10 | 8 ± 4 | 366 |
| XVII | 20 | 20 | 15 ± 6 | 365 |
| XVIII | 2.5 | 10 | 10 ± 4 | 358 |
| XIX | 500 | 5 | NA | NM |
| XX | 0.5 | 10 | 9 ± 4 | 242 |
| XXI | 500 | 5 | NA | NM |
| XIII | 20 | 10 | 10 ± 4 | NM |
| VIII | 500 | 5 | NA | NM |
| I | 20 | 20 | 9 ± 3 | NM |

[a] Average number of cockroaches displaying typical sexual behavior within 3 min in a group containing 25 males ± SD.
[b] NA = not active.
[c] NM = not measured.

provided compounds with significant activities (XVI–XVIII) suggesting that the carbonyl oxygen atom in the ester group is required to induce the activity.

The pheromone activity was shown to be affected by the position of the carbonyl group and length of the substituent (Figure 2). Analogs (III, V, and VI) with a carbonyl group linked directly ($\alpha$-position) to the verbanyl skeleton were inactive. Several of the analogs with the carbonyl group at $\beta$-position via an oxygen atom (XVI–XIX) or a methylene group (VII, X, and XI) exhibited significant or slight activity (2.5- to 100-$\mu$g dose). Analogs having the group at $\gamma$-position (XIII) were also active, while that at the $\delta$-position (VIII) had no activity. Ester XIII, possessing the $\gamma$-carbonyl group, showed an activity comparable to the original mimic I. This means that the receptor site to the carbonyl oxygen atom is able to interact with the oxygen atom at either $\beta$- or $\gamma$-position. The carbonyl oxygen of VIII may be out of range. From these results, the

$\beta$-position of the carbonyl group in ester linkage was concluded to be the most suitable position for generating sex pheromone activity.

Activities observed in X and XVI–XVIII seemed attributable to the electron-donating mesomeric effect of the alcoholic oxygen atoms ($\gamma$-oxygen in X and $\alpha$-oxygen in XVI–XVIII) to the carbonyl oxygen atom. However, oversized length for the receptor space caused inactivity in XIX. On the other hand, as illustrated by the inactivity of VII and XI, the methylene group did not contribute as the electron donor to the carbonyl oxygen atom.

(+)-Verbanyl methylcarbonate (XX) was consequently designed to prepare a more potent sex pheromone mimic, since the carbonyl group of this analog satisfied the conditions induced above ($\beta$-position in the ester group with suitable size and enriched electron density on its oxygen atom). Furthermore, the substituent of XX was composed of structural factors of two active analogs, X and XVII ($-CH_2COOCH_3$ and $-OCOCH_3$, respectively). Methylcarbonate XX, in fact, demonstrated prominent activity at a 0.5-$\mu$g dose, and this is the most potent mimic among the reported sex pheromone mimics of this cockroach (Bowers and Bodenstein, 1971; Tahara et al., 1975; Nishino et al., 1977). However, ethylcarbonate (XXI) lost activity completely because of the bulk of the substituent.

In estimating the magnitude of the electron density on oxygen atoms, the $^{17}O$ chemical shift in [$^{17}O$]NMR spectrum was considered as a simple indicator. In [$^{17}O$]NMR, $\pi$ donor substituents to a carbonyl group displace the $^{17}O$ chemical shift of the carbonyl oxygen [expressed as $\delta(^{17}O)$ (C = O) hereafter] upfield. This effect is cumulative, and when $2\pi$ donors are introduced, as in a carbonate group, the carbonate oxygen resonates further upfield (Klemperer, 1978). If $\delta(^{17}O)$ (C = O) is assumed to depend linearly on $\pi$ bond order, $\delta(^{17}O)$ (C = O)s of aliphatic aldehydes ($\pi$ bond order, 1) at 600 ppm [relative to $H_2O$ ($\pi$ bond order, 0)] is predicted to shift to 200 ppm in the carbonyl oxygen of carbonate ($CO_3^{2-}$, $\pi$ bond order, $\frac{1}{3}$). The observed chemical shift for $CO_3^{2-}$ is 192 ppm, showing good agreement with the predicted value. This upfield shift of $\delta(^{17}O)$ (C = O) is due to the shielding effect of increased electron density on the oxygen atom. Thus $\delta(^{17}O)$ (C = O) is shown to be applicable as an indicator for electron density on the atom.

Because of low natural abundance and appreciable electric quadrupole moment of $^{17}O$, large quantities of sample are required for a well-resolved [$^{17}O$]NMR spectrum. Moreover, $^{17}O$ chemical shift suffers considerable solvent effect by solvents (Sardella and Stothers, 1969; Reuben, 1969; Christ and Diehl, 1963). Hence, in our [$^{17}O$]NMR measurement, neat liquids of isopropyl analogs corresponding to key verbanyl analogs (X, XI, and XVI–XVIII) were employed.

Alkyl groups attached to the alcoholic oxygen atom of the ester group cause no significant effect on $\delta(^{17}O)$ (C = O) [for example, $CH_3OCOCH_3$ (355 ppm) and $CH_3CH_2OCOCH_3$ (356)] (Christ et al., 1961). Furthermore, concerning the alkyl substituent effect on the $^{17}O$ chemical shift of the carbonyl group, alkyl groups further removed than the $\gamma$-position to a ketone group do not influence

the chemical shift value of the ketone oxygen [$CH_3CH_2COCH_3$ (548.5), $CH_3(CH_2)_2COCH_3$ (554) and $CH_3(CH_2)_3COCH_3$ (553 $\pm$ 1)] (Delseth and Kintzinger, 1976). Based on these values, similar $\delta(^{17}O)$ (C = O) were expected between verbanyl and the corresponding isopropyl analogs whose shift values are indicated in Table 1.

Three categories of $\delta(^{17}O)$ (C = O), depending upon the types ($\pi$ bond orders) of carbonyl groups [571 (ketone), around 365 (ester), and 242 ppm (carbonate)], indicated the potency of the activity [inactive ketone (XI), active esters (X and XVI–XVIII) and more active carbonate (XX), respectively]. It was thus verified that the upfield shift of $\delta(^{17}O)$ (C = O) expressed the increase of electron density on the carbonyl oxygen atom which is supposed to cause the increase of pheromonal activity. In this case, the carbonyl oxygen atom distant from the neighboring groups, $-\underline{CH_2}-(C=O)-$ and $-\underline{O}-(C=O)-$, seems not to be hindered sterically by the groups, although $-CH_2-$ is larger than $-O-$ (cf. Taft's steric parameter).

Although alcohol oxygen atoms resonate at a much higher field than that of carbonyl oxygen atoms in [$^{17}O$]NMR (Christ et al., 1961) (namely, electron density: $-OH > C=O$), the formate (XVI) (R = OCOH) was pheromonally active, whereas the parent alcohol (R = $CH_2CH_2OH$) of VIII was inactive. Accordingly, the ester carbonyl oxygens in the mimics are expected to play a role of a softer ligand than the alcohol (OH) oxygens and to attach to soft electron acceptors in the receptor. Esters X and XVI–XVIII showed similar $\delta(^{17}O)$ (C = O)s despite having different pheromone activities. This suggests that pheromone activity depends upon not only electron density of the carbonyl oxygen atom but also the existence of the alkyl group of the ester group, which would be required to regulate molecular size to the receptor space ($H < OCH_3 < CH_3 < CH_2CH_3$ in Taft's Es, steric substituent constant) and/or required to interact hydrophobically with a second site of the receptor [$OCH_3 \leqslant H < CH_3 < CH_2CH_3$ in hydrophobicity parameter ($\pi$)].

Since we revealed that the verbanyl sex pheromone mimics participated in the sex pheromone receptor for the genuine sex pheromone, periplanone-B (Nishino and Manabe, 1983), the ester carbonyl group of the mimics is presumed to correspond structurally to the ketone group of periplanone-B (Persoons et al., 1979). In fact the two carbonyl oxygens overlapped without interference when a structural overlap between a verbanyl mimic (XVIII) and periplanone-B was inspected with a Dreiding model considering the other important structural factors of XVIII (Nishino and Takayanagi, 1981; Takayanagi and Nishino, 1982; Nishino et al., 1982, 1984; Manabe et al., 1983). Nevertheless, a large difference of activity was observed between XX and periplanone-B [$1 \times 10^{-5}$ $\mu g$ (Nishino and Kuwabara, 1983) and $1 \times 10^{-6\sim7}$ $\mu g$ (Adams et al., 1979)]. Periplanone-B contains other polar functional groups (double bonds and two epoxides) which may also serve to interact with other sites of the receptor, whereas XX possesses only carbonyl oxygen. This may be a reason for the much stronger activity of periplanone-B than XX.

Although our trial for relating biological activity with a $^{17}O$ chemical shift is with a limited number of samples, [$^{17}O$]NMR seems useful for structure–activity studies in biologically active compounds possessing oxygen atoms which are revealed as active sites in the molecule.

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# OLFACTORY APOSEMATISM
## Association of Food Toxicity with Naturally Occurring Odor

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**Abstract**—Visual and acoustic cues may serve as aposematic signals that warn predators of poisonous foods. Olfactory aposematism, the use of innocuous odors as warning signals for toxic foods, is another possible means of alerting an animal that a potential food item is unpalatable. Although it has been suggested that olfactory aposematism might be the principle mode of warning utilized by plants in their defense against herbivores, experimental evidence is lacking. This study demonstrates that the opossum, *Didelphis virginiana,* can utilize an innocuous volatile compound found naturally in a mushroom as a warning signal for a delayed illness caused by mushroom toxin. This supports the contention that characteristic odors of toxic plants may serve a warning function, protecting herbivores from being poisoned and plants from being consumed.

**Key Words**—Aposematism, olfactory, odor, opossum, *Didelphis virginiana,* 1-octen-3-ol, mushroom volatile, toxicity, bioassay.

## INTRODUCTION

All animals must choose from a variety of foods, some of which contain toxins. Those which are distasteful are generally quickly rejected by an animal. Other foods, possessing palatable toxins with delayed systemic actions, may not be rejected initially. Many vertebrates, however, can learn to avoid such toxic foods and are thus spared repeated, potentially lethal, "taste trials." Visual signals may serve as the aposematic cues that an animal associates with noxious prey. For example, vertebrates develop learned food aversions to the conspicuous visual cues of toxic monarch butterflies (Brower, 1969), bees (Brower et al., 1960; Brower and Brower, 1962), and coral snakes (Smith, 1977).



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Although visual aposematism has been most extensively studied, acoustic and olfactory signals may also warn potential predators of unacceptable foods. For example, the buzz of venomous wasps deters potential amphibian predators (Brower and Brower, 1965). Cott (1940) suggested that specific noxious odors may alert predators and warn them of the toxicity of potential food items. Many mammals and insects emit volatile substances that act as both a warning advertisement and a noxious defense. Rothschild (1961) reported that "scent patterns" could act as "warning mechanisms for color-blind predators" but also presented examples in which the odor itself was distinctly noxious and thus served both a warning and defensive function.

One might predict, however, that many innocuous odorants could act as warning signals alerting foraging animals of toxins, in the same manner that the totally innocuous color patterns of various insects and snakes are aposematic. Indeed, Palmerino et al. (1980) demonstrated that when a poisoned food with a characteristic odor and taste is fed to rats, they become ill and the "odor acquires some of the memorial properties of a taste." Applying these findings to the realm of plant–herbivore interactions, Eisner and Grant (1981) suggested that olfactory aposematism might be the principle mode of warning utilized by plants; however, experimental evidence for this concept is lacking.

In the present study, I present evidence that the opossum, *Didelphis virginiana,* is able to use an innocuous volatile compound found naturally in a fungus as a warning signal of a delayed illness. The development of such an aversion towards a volatile odorant may be a means by which opossums and other animals learn to avoid toxic plants and fungi.

The relative concentrations of volatile components varies considerably among different species of mushroom. One of these volatiles, 1-octen-3-ol, imparts to fungi the characteristic odor and taste that we associate with mushrooms (Pyysalo, 1976). Opossums can readily distinguish the various wild mushrooms presented in feeding tests (Camazine et al., 1983), and thus it seemed possible that the animals were choosing on the basis of olfactory cues. Could the opossums therefore distinguish commercial mushrooms treated with an odorless and tasteless mushroom toxin (muscimol) from commercial mushrooms to which 1-octen-3-ol was added? Furthermore, after becoming ill from the toxin, would the opossums develop an aversion to the mushrooms flavored with octen-3-ol and reject these flavored mushrooms without tasting them?

Octen-3-ol is not intrinsically noxious but provides the opossum with an olfactory cue which it subsequently associates with a delayed illness induced by the mushroom toxin, muscimol. Blends of volatile substances, too subtle for humans to distinguish, may be the specific cue that allows the opossum to differentiate among the many potentially toxic food items that it encounters on its nightly feeding forays.

## METHODS AND MATERIALS

*The Test Animal—The Virginia Opossum.* Opossums are omnivores that eat fungi in the wild (Fogel and Trappe, 1978). In the laboratory, they will avoid a number of chemically protected mushrooms; certain pungent fungi are rejected immediately after tasting (Camazine et al., 1983). Toxic *Amanita muscaria* mushrooms, which are initially palatable but induce vomiting, are refused after one or two feeding sessions and continue to be avoided for periods of up to a year (Camazine, 1983; Camazine, unpublished observations). The nocturnal habits and keen olfactory sense of opossums (Moulton, 1973) suggest that olfactory signals may be important cues which these animals utilize in the evaluation and subsequent rejection of foods such as the *Amanita* mushrooms. After an opossum has learned to avoid a particular noxious mushroom, the animal subsequently rejects the fungus without tasting it. This suggests that a food odor which is not intrinsically repellent may later act as an important cue in the evaluation and rejection of a food with a delayed toxic effect.

*Opossum Feeding Bioassy.* In previous studies using the opossum feeding bioassay (Camazine, 1983; Camazine et al., 1983), it had been found that opossums develop an aversion to a novel, nonpoisonous mushroom when it is eaten in conjunction with the mushroom toxin, muscimol, which is an emetic. The aversion develops regardless of whether the toxin is added directly to the novel mushroom or to a familiar control item.

Since the opossums developed an aversion only towards the unfamiliar mushrooms, it appeared that they were associating the illness with the novelty of the wild mushrooms. Whether the color, odor, taste, or combination of these characteristics served as the warning (conditioning) stimulus was not ascertained in the earlier studies. The following experiment was therefore designed to determine whether olfactory cues could be used to assess the novel fungi and whether they were serving as olfactory aposematic signals for the delayed illness induced by the toxin.

Four opossums that readily accepted the commercial mushroom *Agaricus bisporus* were used (three female and one male animal, ages 2–3 years, born in captivity or obtained as young from the pouches of their mothers). Their diet consisted of commercial dog food and water. The animals were tested once daily just prior to their regular feeding time to assure that they were uniformly hungry. In the feeding bioassay, individual opossums were offered fresh pieces (approximately $2 \times 2 \times 2$ cm) of a given test mushroom and comparable pieces of an edible control mushroom. Mushroom pieces were presented one at a time in three-item sequences consisting of two pieces of the test mushroom and one randomly interspersed piece of the control mushroom. Each item was left with the animal for a maximum of 30 sec before being removed and the next item

presented. A total of 27 items (test plus control) were presented to each opossum per session. If an item was totally consumed, it was scored as eaten; if it was partially eaten, rejected on close inspection (tasted, sniffed, and/or manipulated), or ignored from a distance, it was scored as rejected. If an item at the end of a session was rejected, it was not tallied since the failure to eat might have been due to satiation of the animal. For each opossum a palatability rating for the test and control mushrooms was calculated as the percent of the items eaten.

In the present experiments, the experimental items consisted of morsels of the commercial *Agaricus bisporus* mushroom divided into two groups. The first group, poisoned items, consisted of morsels of *Agaricus* to which 1 mg of muscimol was topically applied in the conditioning trials on days 1 and 2. The second group, flavored items, comprised morsels of *Agaricus* to which 2 $\mu$l of 1-octen-3-ol was applied. The flavored items were not poisoned with muscimol. After the two conditioning trials on days 1 and 2, there was a final trial four days later (day 6) at which time no poison was administered. However, if an animal developed a total aversion to the flavored mushroom on day 2 after the single conditioning trial on the previous day, the animal was not tested on day 6; if it continued to eat the poisoned mushroom on day 2 without consuming any of the flavored mushroom, it would be unable to associate the resulting illness with the flavored mushroom.

It was also necessary to demonstrate that the proposed innocuous olfactory cue was not in itself noxious and was thus incapable of inducing an aversion. Therefore the identical feeding trials were repeated three months later with opossums 1 and 2, except that in these trials no muscimol toxin was added to the mushrooms.

## RESULTS

The results of the feeding tests are illustrated in Figure 1. In all the trials the poisoned mushroom was completely eaten by all the animals. On day 1, the flavored mushroom (*Agaricus* with the volatile odorant, 1-octen-3-ol) was also highly acceptable (mean palatability rating = 86.8 $\pm$ 15.7%). Within 25 min of the completion of the feeding trials, all the animals became ill and vomited.

On the second conditioning trial (day 2) muscimol was again added to the *Agaricus* morsels. Three of the four opossums continued to consume the flavored mushroom pieces, but opossum 4 rejected them without tasting. As in previous experiments (Camazine, 1983) individual opossums varied with respect to whether one or two conditioning trials with a gastrointestinal poison were needed to induce a food aversion.

On day 6 the experiment was repeated without muscimol. As expected the three animals that were tested now showed a marked aversion to the flavored

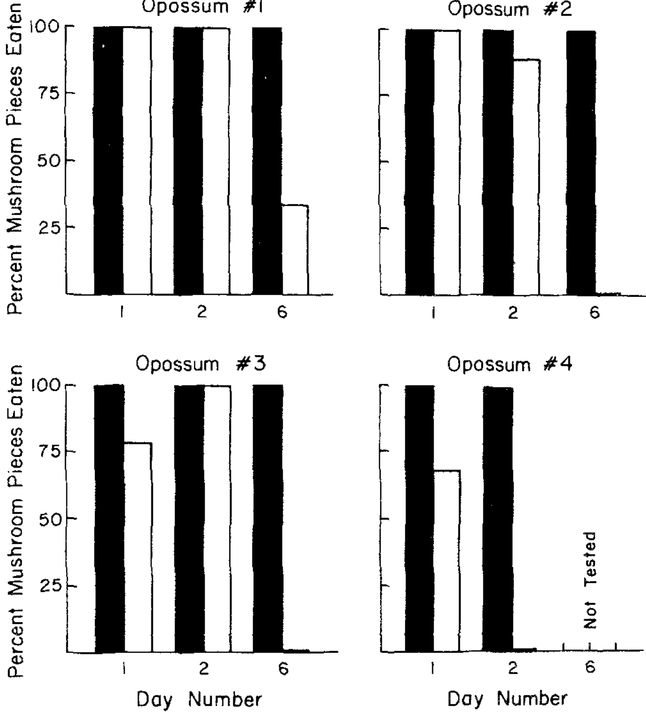


FIG 1. The palatability of the nonflavored mushroom (*Agaricus bisporus* with muscimol) shown with solid bars versus that of the flavored mushroom (*Agaricus bisporus* with octen-3-ol) shown with open bars, during three trials spanning six days. On days 1 and 2 the mushroom toxin, muscimol, was added to the nonflavored mushroom pieces. On day`6, no toxin was added to this group. Data are expressed as percent total mushroom pieces eaten by each of the four opossums.

mushrooms, rejecting them without tasting; each animal would approach the flavored mushroom morsel, inspect it closely with its muzzle and move away from the item. The mean palatability rating of the flavored items was 11 ± 19.1%. (The fourth opossum who had developed a total aversion to the flavored mushrooms on day 2 was not retested on day 6.) For animals 1, 2, and 3 (which required two conditioning trials to develop a food aversion), there were significant differences between the numbers of flavored items eaten on day 1 and the number of flavored items eaten on day 6 ($P < 0.01$, paired $t$-test). For animal 4 (in which a food aversion developed after the single conditioning trial), there were significant differences between the numbers of flavored pieces eaten on days 1 and 2 ($P < 0.01$).

In the experiment three months later, which was a control for the possible aversive effects of 1-octen-3-ol, no muscimol was added to the mushroom pieces.

The animals ate 100% of both the flavored and control pieces on all three trials on days 1, 2, and 6.

## DISCUSSION

These experiments demonstrate that an innocuous volatile compound found naturally in a mushroom can act as an olfactory warning signal for the delayed illness induced by a mushroom toxin. Subtle blends of volatile components in foods may act as warning signals for many animals that rely on olfaction in the evaluation of food. This may be particularly true of nocturnal animals such as the opossum. Similar flavor–illness aversions have been demonstrated experimentally in the rat (Palmerino et al., 1980).

That plants and fungi might possess olfactory warning signals is not surprising and is entirely analogous to the more familiar visual aposematism seen in many brightly colored insects such as the toxic monarch butterfly, and the acoustic aposematism shown by buzzing wasps and perhaps by arctiid moths which emit ultrasonic cries when approached by foraging bats (Dunning and Roeder, 1965; Fullard, 1977).

An additional interesting feature of these results is the lack of any residual effect when the animals were retested three months later; all of the 1-octen-3-ol flavored and unflavored mushrooms were eaten. This would suggest either that the test involved rapid extinction of the learned aversion or a forgetting of the pairing of the novel olfactory cue with muscimol which occurred three months earlier. One explanation may be that olfactory stimuli are less effective than taste stimuli, a topic addressed by Garcia and Rusiniak (1980). One might also hypothesize that olfactory cues alone are not a primary means of conditioning a food aversion in nature, but that "flavor aposematism," involving both taste and odor cues may be more significant and durable in the natural setting.

The fact that the naturally occurring toxin muscimol is hallucinogenic for humans further suggests a role in nature for the many hallucinogenic compounds found in plants and fungi (Camazine, 1983). A wide variety of psychoactive compounds can be used to induce taste aversions in animals (Gamzu, 1977) and the natural occurrence of hallucinogenic compounds may be a reflection of their role as defensive agents against herbivorous (and fungivorous) animals.

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# ISOLATION AND IDENTIFICATION OF MOSQUITO[1] REPELLENTS IN *Artemisia vulgaris*[2]

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**Abstract**—The mugwort *Artemisia vulgaris* L. (Compositae: Anthemideae) contains insect repellents which can be released from the plant tissues by combustion. Work was carried out to isolate and identify the repellent compounds. The dried, pulverized whole plants were steam-distilled to give a repellent essential oil which was fractionated by column chromatography. Active fractions were analyzed by capillary GC and by combined GC-MS. A number of compounds, mainly monoterpenoids, were identified. When tested as repellents against the yellow fever mosquito *Aedes aegypti* L. (Diptera: Culicidae), (±)-linalool, (±)-camphor, (+)-camphor, (−)-camphor, isoborneol, (−)-borneol, terpinen-4-ol, and isobornyl acetate were active at 0.14 mg/cm$^2$ or higher. Nonanone-3, ($\alpha$ + $\beta$)-thujone, and bornyl acetate were active at 0.28 mg/cm$^2$ or higher. $\beta$-Pinene, myrcene, $\alpha$-terpinene, (+)-limonene, and cineole were active at 1.4 mg/cm$^2$. Of the repellent compounds identified, terpinen-4-ol was the most active and was as effective as dimethyl phthalate.

**Key Words**—*Artemisia vulgaris*, mosquito repellents, *Aedes aegypti*, Diptera, Culicidae, linalool, camphor, isoborneol, borneol, terpinen-4-ol, isobornyl acetate.

## INTRODUCTION

The mugwort *Artemisia vulgaris* L. (Compositae: Anthemideae) is a rampant, perennial weed found in Europe, Asia, and the United States. In the United

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States, several varieties of this weed occur at higher elevations in California, Montana, Colorado, and other western states. It is frequently used in folk medicine in India for its various pharmacological properties (Kundu et al., 1969). In China, *A. vulgaris* is used in traditional herb medicine. Old leaves of this weed are dried and made into a moxa (soft wooly mass) which is used as a cautery (burning or searing) by being ignited on the skin. In Chinese cuisine, young leaves of this weed can be added to rice cakes or dumplings to impart a pleasant aroma.

It had come to our attention that *A. vulgaris* possessed insect-repellent activity (Prof. C.S. Tsi, private communication, 1982). Bundles of the dry weed are slowly burned to produce smoke which is reportedly repellent to hematophagous insects. This method of repelling biting insects, particularly in rural areas of China, has been practiced for centuries. This approach is similar to the use of pyrethroid-containing mosquito incense sticks or mosquito coils commonly practiced in Asian countries today.

In our preliminary studies, a benzene–methanol extract of *A. vulgaris*, when applied on human skin, showed repellency against the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae) (Hwang, Mulla, and Axelrod, unpublished data). This finding was significant because it was the extract of the plant, not the smoke, that exhibited repellency. It also prompted us to embark upon chemical and biological investigations in search of repellent compounds. Here we report the isolation and identification of mosquito-repelling compounds present in the shrub *A. vulgaris*.

## METHODS AND MATERIALS

*A. vulgaris* was collected at the Ma-lian-wa Experiment Station, Chinese Academy of Medical Sciences, Beijing, China, or purchased from local herb shops in Beijing. The collected weed was identified as *A. vulgaris*, air-dried, and shipped to the United States. Upon receipt, the dry herb was pulverized in a Wiley mill (A.H. Thomas Co., Philadelphia, Pennsylvania), and the powder thus obtained was stored in a freezer until use.

The *A. vulgaris* powder (200 g) was macerated in water (1.5 liter) overnight and steam-distilled until no more volatile substance was obtained. The distillate was extracted three times with benzene, and the benzene extracts were combined and dried over $Na_2SO_4$. Evaporation of the solvent gave the *Artemisia* essential oil (0.9 g). The remaining steam-distillation residue was filtered to remove the plant material, and $\frac{1}{5}$ of the aqueous filtrate was extracted three times with benzene. The benzene extracts were combined and dried over $Na_2SO_4$. Evaporation of the solvent gave a dark green oil (0.5 g). About $\frac{1}{6}$ of the plant material was extracted with a mixture of benzene and methanol (3 : 1, 300 ml) at room temperature with occasional stirring for 24 hr. The plant material was

removed by filtration, and the benzene–methanol solution was dried over $Na_2SO_4$ and distilled to give a dark brown oil (0.8 g). This procedure was repeated several times to accumulate enough of the essential oil for fractionation and biological studies.

The *Artemisia* essential oil (1.8 g) was chromatographed on a Florisil column (100 g, 100 × 2.5 cm) and eluted successively with (1) hexane (1175 ml), (2) hexane–benzene (1 : 1, 700 ml), (3) benzene (600 ml), (4) benzene–acetone (1 : 1, 900 ml), (5) acetone (400 ml), (6) acetone–methanol (1 : 1, 900 ml), and (7) methanol (650 ml). Aliquots of 25 ml were collected up to fraction 61; thereafter aliquots of 50 ml were collected. The solvents were evaporated from these aliquots, and the number of the fractions was plotted against the weight in each fraction (Figure 1). The fractions obtained in sufficient quantities were bioassayed for repellency.

The active fractions were analyzed with a Packard model 803 FID gas chromatograph equipped with a 60-m × 0.25-mm-ID silica capillary column

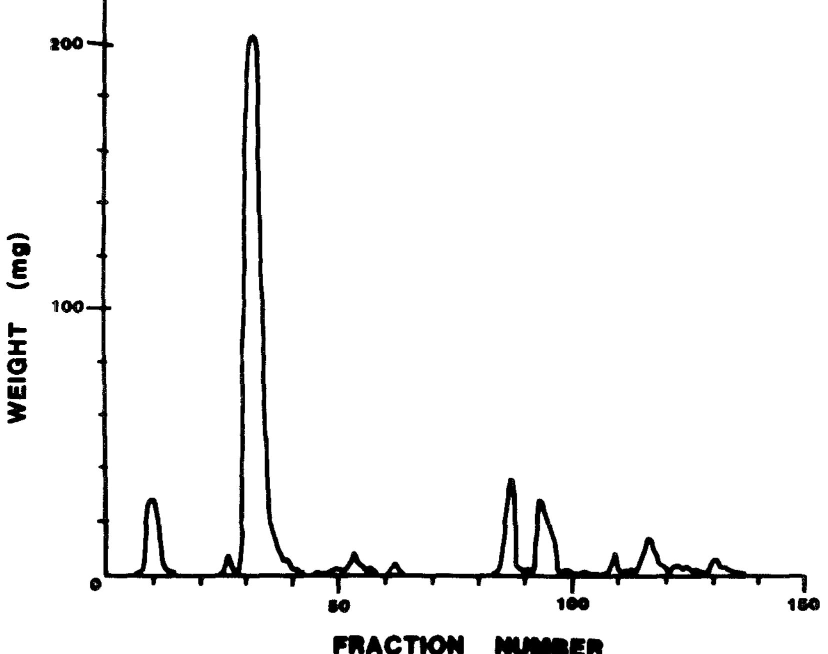


Fig. 1. Gravimetric column chromatogram of the *Artemisia* essential oil on a Florisil column. The following solvents were used: fractions 1–47, hexane; fractions 48–68, hexane–benzene (1 : 1); fractions 69–81, benzene; fractions 82–99, benzene–acetone (1 : 1); fractions 100–108, acetone; fractions 109–125, acetone–methanol (1 : 1); fractions 126–138, methanol.

coated with DB-5 (bonded SE-54). The column temperature was programed from 45 to 150°C at a rate of 1°C/min. GC-MS analysis was conducted with a Finnigan model 1015 mass spectrometer interfaced with a Varian Aerograph model 1400 gas chromatograph and a System 150 data acquisition and reduction system. A 10-ft × $\frac{1}{4}$-in.-ID stainless-steel column packed with 20% LAC-2R446 on 60–80 mesh Chromosorb W was used in the GC-MS analysis. The column temperature was programed from 50 to 150°C at a rate of 1°C/min after an initial isothermal period of 50 min at 50°C.

An olfactometer was devised for determining the repellency of the various fractions obtained during the isolation procedure and the various compounds identified during the GC and GC-MS analyses. The olfactometer, placed in a constant temperature room at 23.5 ± 1°C, consisted of a cylindrical plastic food container (11 cm diameter, 7.5 cm high) with a circular screen window (4 cm diameter) in the side. A vacuum line was attached to the container to create an air flow from the window into the olfactometer at 1 liter/min. Twenty-five, 6 to 8-day-old, non-blood-fed, adult female *A. aegypti* were placed in the olfactometer. A predetermined volume of an acetone or ethanol solution of a fraction or compound was applied onto an area of 6 × 6 cm in the palm of the hand of an experimenting human subject. After evaporation of the solvent, the palm was cupped over the screen window of the olfactometer but kept several millimeters away from the screen. In this way, the mosquitoes would probe through the screen in an attempt to feed but could not reach the palm to do so. Prior to testing the treated palm, an untreated palm (solvent only) was also exposed to mosquitoes in the same manner and considered as a control. The numbers of the mosquitoes landing on the screen and then probing at the control and treated palms were counted after 1 min. The same procedure was repeated 6, 12, or 18 times in a test for replication.

In the bioassay tests, commonly used insect repellents, DEET (*N,N*-diethyl-*m*-toluamide) and DMP (dimethyl phthalate), were also tested for comparison.

Chemicals for bioassay tests were obtained from commercial sources; they were at least of 90% purity and used without further purification.

The degree of repellency was expressed in terms of percent repellency calculated according to the following formula:

$$\%\ \text{Repellency} = \frac{N_c - N_t}{N_c} \times 100$$

wherein $N_c$ denotes the mean number of mosquitoes landing on the screen and probing at the control palm, and $N_t$ denotes that at the treated palm. The significance of the differences between the treated and the control was determined by the Student's $t$ test.

## RESULTS AND DISCUSSION

The *Artemisia* essential oil showed 91% repellency (0.4 mg/cm$^2$) against female *A. aegypti*, whereas the dark green oil obtained from the aqueous portion of the steam-distillation residue showed only 33% repellency. The dark brown oil obtained from the plant material of the steam-distillation residue exhibited no repellency. It was thus apparent that the repellent compounds were volatile and hence steam-distillable.

Chromatography of the *Artemisia* essential oil on the Florisil column gave a total of 138 fractions. Fractions 30–35, which were eluted with hexane, formed a major peak, and a number of minor peaks spread widely over the chromatogram (Figure 1). Fractions or combinations of fractions, upon bioassay tests, showed various levels of repellency (Table 1). Except for fractions 9–13, all other fractions showed significant repellency over the control, and the level of the repellency was comparable to that shown by DEET or DMP. For GC and GC-MS analyses, fractions 30–34 were used because of their larger quantities. GC analysis of fractions 30–34 indicated that they had similar compositions.

A typical gas chromatogram of combined fractions 33 and 34 in isooctane on a DB-5 capillary column is shown in Figure 2. $\alpha$-Pinene was added as in-

TABLE 1. PERCENT REPELLENCY OF VARIOUS COLUMN-CHROMATOGRAPH FRACTIONS OF *Artemisia* ESSENTIAL OIL AGAINST *Aedes aegypti*

| Fraction No.[a] | Mean No.[b] | | |
|---|---|---|---|
| | Control | Treated | % Repellency[c] |
| 9–13 | 12.83 ± 1.71 | 11.67 ± 1.09 | 6.28 ± 21.02 |
| 30 | 21.33 ± 2.07 | 3.50 ± 1.31 | 82.34 ± 8.30* |
| 31 | 20.67 ± 2.00 | 1.00 ± 1.34 | 93.73 ± 6.27* |
| 32 | 15.17 ± 2.65 | 2.17 ± 1.17 | 83.88 ± 10.56* |
| 33 | 13.83 ± 1.86 | 2.83 ± 1.92 | 77.88 ± 17.53* |
| 50–59 | 11.33 ± 1.87 | 1.00 ± 0.72 | 89.85 ± 8.03* |
| 86–87 | 11.00 ± 1.13 | 1.33 ± 1.58 | 85.26 ± 14.74* |
| 93–96 | 11.67 ± 2.66 | 2.17 ± 1.28 | 77.77 ± 16.09* |
| 106, 107 | 18.17 ± 2.65 | 7.00 ± 2.37 | 58.70 ± 38.13* |
| 108 | 15.67 ± 2.75 | 7.00 ± 0.88 | 52.87 ± 13.91* |
| DEET | 18.83 ± 3.42 | 4.08 ± 2.73 | 76.11 ± 17.54* |
| DMP | 10.16 ± 1.24 | 0.50 ± 0.40 | 94.57 ± 4.55* |

[a] In each preparation, 10% ethanol solution of a fraction or a combination of fractions was made, and an aliquot of 0.1 ml (0.2 ml for fraction 33) was applied onto 6 × 6 cm of human palm.

[b] Mean numbers with 95% confidence intervals of mosquitoes landing and probing based on six replicates.

[c] Percent repellency with 95% confidence intervals followed by an asterisk denotes that the difference between treated and control is significant at the 1% level in the Student's *t* test.

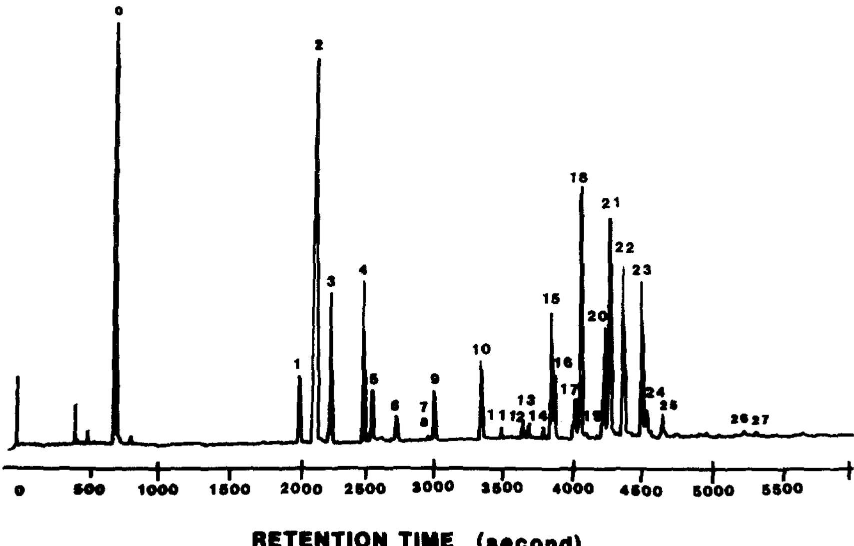


FIG. 2. Typical capillary gas chromatogram of fraction 33 from the *Artemisia* essential oil on a DB-5 column. The peak numbers correspond to those in Table 2.

ternal standard. Identification was made by comparing the retention times of the unknown peaks with those of authentic compounds and/or by the mass spectrum of each peak after being separated by LAC-2R446 column in the GC-MS system. Compounds thus identified, together with their methods of identification, are listed in Table 2.

The compounds identified were mostly cyclic or acyclic monoterpenes and their derivatives, commonly found in essential oils of various plants. Rao and Sood (1964) reported that freshly collected leaves and flowers of *A. vulgaris* were steam-distilled to yield a dark green oil which contained *p*-cymene, linalool, β-thujone, azulene, and β-thujyl alcohol. Except for azulene, all other monoterpenes were found in fractions 30–34 in our present study (Azulene was found in fractions 28 and 29). Kundu et al. (1969) detected the presence of α-thujone and α- and β-pinenes together with other triterpenoids in *A. vulgaris*. Nano et al. (1976, 1977) isolated camphor, β-thujone, and myrcene from mature plants of this species. Compounds listed in Table 2, except those found by the above-mentioned workers, are for the first time isolated and identified in *A. vulgaris*.

Although the essential oil of *A. vulgaris* was first analyzed in detail in this study, oils from other plants belonging to the genus *Artemisia* have been widely investigated. Examples are *A. californica* (Halligan, 1975), and *A. douglasian* Scora et al., 1984).

Most of the compounds identified were bioassayed for their repellency

TABLE 2. COMPOUNDS IDENTIFIED FROM FRACTIONS 33 AND 34 OF *Artemisia* ESSENTIAL OIL

| Peak No.[a] | Compound | Method of identification | |
|---|---|---|---|
| | | GC | GC-MS |
| 0 | Isooctane (solvent) | | |
| 1 | Tricyclene | + | |
| 2 | α-Pinene | + | |
| 3 | Camphene | + | |
| 4 | β-Pinene | + | |
| 5 | Myrcene | + | + |
| 6 | α-Terpinene | + | + |
| 7 | *p*-Cymene | + | + |
| 8 | Limonene | + | + |
| 9 | Cineole | + | + |
| 10 | Artemisia ketone | + | + |
| 11 | Nonanone-3 + α-thujone | + | + |
| 12 | Linalool | + | + |
| 13 | β-Thujone | + | + |
| 14 | Thujyl alcohol (isomer) | + | + |
| 15 | Artemisia alcohol | + | + |
| 16 | Unknown | | |
| 17 | Thujyl alcohol (isomer) | + | + |
| 18 | Camphor | + | + |
| 19 | Isoborneol | + | + |
| 20 | Unknown | | |
| 21 | Borneol | + | + |
| 22 | Terpinen-4-ol | + | + |
| 23 | Unknown | | |
| 24 | Unknown | | |
| 25 | Unknown | | |
| 26 | Isobornyl acetate | + | + |
| 27 | Bornyl acetate | + | + |

[a] The peak numbers correspond to those in Figure 2.

against *A. aegypti*, and the bioassay results are listed in Table 3. All compounds tested showed significant repellency at the highest dosage of 1.4 $mg/cm^2$, with (+)-limonene, linalool, camphor, isoborneol, and terpinen-4-ol exhibiting about the same degree of activity as DMP at the same dosage.

At a lower rate of 0.28 $mg/c^2$, (+)-limonene lost its repellency, but the rest of the compounds remained active. (+)-Camphor and terpinen-4-ol were still as active as DMP, and (±)-camphor, isoborneol, and bornyl acetate were very active, showing more than 80% repellency.

Although linalool, (±)-camphor, (+)-camphor, (−)-camphor, isoborneol, (−)-borneol, terpinen-4-ol, and isobornyl acetate were all significantly repellent

TABLE 3. PERCENT REPELLENCY OF COMPOUNDS IDENTIFIED

| Peak No. | Compound | Dosage ($mg/cm^2$)[a] 0.14 Mean No.[b] Control | Treated | % Repellency[c] |
|---|---|---|---|---|
| 4 | β-Pinene | 14.17 ± 1.71 | 12.83 ± 1.99 | 6.37 ± 25.31 |
| 5 | Myrcene | 12.17 ± 2.35 | 9.17 ± 1.18 | 19.82 ± 25.11 |
| 6 | α-Terpinene | 17.33 ± 1.10 | 14.50 ± 1.66 | 15.41 ± 14.92 |
| 8 | (+)-Limonene | 7.83 ± 1.71 | 6.33 ± 1.20 | 11.65 + 34.69 |
| 9 | Cineole | 10.67 ± 2.56 | 11.67 ± 2.28 | −21.45 ± 50.57 |
| 11 | 3-Nonanone | 12.92 ± 0.89 | 11.00 ± 1.69 | 13.53 ± 19.01 |
| 11, 13 | (α + β)-Thujone | 10.08 ± 1.70 | 7.42 ± 2.55 | 19.91 ± 38.75 |
| 12 | (±)-Linalool | 8.67 ± 1.40 | 2.67 ± 0.85 | 66.82 ± 15.09* |
| 18 | (±)-Camphor | 9.50 ± 1.01 | 5.83 ± 1.90 | 35.73 ± 26.78* |
| 18 | (+)-Camphor | 11.92 ± 0.89 | 7.50 ± 2.23 | 35.31 ± 23.52* |
| 18 | (−)-Camphor | 9.75 ± 1.32 | 4.83 ± 1.85 | 46.92 ± 26.16* |
| 19 | Isoborneol | 11.83 ± 0.93 | 2.50 ± 0.75 | 78.25 ± 7.95* |
| 21 | (−)-Borneol | 11.83 ± 0.87 | 3.50 ± 0.51 | 69.96 ± 6.51* |
| 22 | Terpinen-4-ol | 12.74 ± 1.51 | 2.00 ± 1.02 | 83.13 ± 10.00* |
| 26 | Isobornyl acetate | 11.58 ± 0.70 | 6.33 ± 1.68 | 44.26 ± 17.87* |
| 27 | Bornyl aceate | 13.66 ± 1.65 | 11.33 ± 1.31 | 14.65 ± 19.90 |
| | DEET | – | – | – |
| | DMP | 9.75 ± 0.10 | 1.16 ± 0.63 | 87.31 ± 7.76* |

[a] Various dosages were applied to 6 × 6 cm of human palm.
[b] Mean numbers with 95% confidence intervals of mosquito landing and probing. Based on 6, 12, or 18 replicates.
[c] Percent repellency with 95% confidence intervals followed by an asterisk denotes that the difference between treated and control is significant at the 1% level in the Student's *t* test.

at the lowest rate of 0.14 $mg/cm^2$, only terpinen-4-ol was as active as DMP at this dosage. As the dosage decreased from 1.4 to 0.28 to 0.14 $mg/cm^2$, the percent repellency of terpinen-4-ol changed from 91, 94, to 83 as compared with that of DMP which dropped from 95, 95, to 87. It is reasonable to conclude that the repellencies of terpinen-4-ol and DMP were comparable in our bioassay tests. It is therefore evident that the shrub *A. vulgaris* contains various monoterpenoids which vaporize into the air and repel blood-sucking insects when the plant is slowly burned. This paper provides data to show the repellent activity of the monoterpenoids isolated and identified from this species of plant.

Essential oils are known to show insect repellency, and citronella oil in particular has been used as a mosquito repellent. Inazuka (1982) found that oils of Japanese mint (*Mentha arvensis*) and spearmint (*Mentha spicata*) were re-

FROM *Artemisia* ESSENTIAL OIL AGAINST *Aedes aegypti*

| Dosage (mg/cm²)[a] | | | | | |
|---|---|---|---|---|---|
| 0.28 | | | 1.4 | | |
| Mean No.[b] | | | Mean No.[b] | | |
| Control | Treated | % Repellency[c] | Control | Treated | % Repellency[c] |
| 10.42 ± 0.66 | 10.67 ± 0.74 | −3.21 ± 13.59 | 10.25 ± 1.23 | 3.92 ± 1.09 | 59.91 ± 15.49* |
| 9.08 ± 0.85 | 8.00 ± 0.91 | 10.23 ± 18.43 | 8.92 ± 1.42 | 1.58 ± 1.04 | 79.88 ± 14.80* |
| 10.92 ± 1.12 | 9.75 ± 0.85 | 8.94 ± 17.00 | 8.33 ± 1.04 | 3.33 ± 1.01 | 57.83 ± 17.29* |
| 7.33 ± 0.97 | 4.66 ± 1.58 | 32.33 ± 30.44 | 7.66 ± 1.31 | 0.50 ± 0.97 | 88.43 ± 11.57* |
| 9.17 ± 1.15 | 9.25 ± 0.84 | −3.73 ± 22.24 | 10.42 ± 1.09 | 2.00 ± 0.54 | 80.05 ± 7.30* |
| 11.58 ± 0.98 | 4.58 ± 2.14 | 58.55 ± 21.94* | 9.33 ± 1.98 | 3.00 ± 0.91 | 64.23 ± 17.29* |
| 16.42 ± 5.46 | 2.83 ± 0.99 | 78.37 ± 13.22* | 9.17 ± 1.77 | 1.08 ± 0.82 | 85.93 ± 11.60* |
| 9.08 ± 2.17 | 2.08 ± 1.24 | 72.28 ± 20.25* | 8.75 ± 1.58 | 0.50 ± 0.50 | 92.20 ± 7.12* |
| 10.11 ± 1.06 | 1.78 ± 0.56 | 81.61 ± 7.46* | 9.92 ± 2.52 | 0.92 ± 0.89 | 87.72 ± 12.03* |
| 16.17 ± 4.66 | 1.17 ± 1.10 | 89.97 ± 9.69* | 6.75 ± 0.91 | 0.50 ± 0.50 | 88.42 ± 8.97* |
| 9.08 ± 1.38 | 1.92 ± 0.62 | 77.38 ± 10.19* | 7.08 ± 1.22 | 0.08 ± 0.17 | 97.87 ± 2.13* |
| 9.38 ± 1.29 | 1.72 ± 0.38 | 80.76 ± 6.69* | 12.33 ± 0.61 | 0.50 ± 0.38 | 95.78 ± 3.29* |
| 7.42 ± 1.44 | 1.83 ± 0.72 | 72.41 ± 15.04* | 8.08 ± 1.50 | 1.33 ± 0.56 | 81.59 ± 10.27* |
| 10.17 ± 1.49 | 0.58 ± 0.45 | 93.51 ± 5.37* | 8.33 ± 1.24 | 0.67 ± 0.61 | 90.74 ± 8.63* |
| 8.75 ± 0.91 | 2.67 ± 0.70 | 68.37 ± 11.22* | 8.83 ± 0.99 | 3.83 ± 0.90 | 54.92 ± 15.24* |
| 13.83 ± 1.07 | 2.50 ± 1.32 | 81.11 ± 10.97* | 13.33 ± 0.97 | 4.00 ± 0.51 | 69.59 ± 6.00* |
| – | – | – | 18.83 ± 2.42 | 4.08 ± 2.73 | 76.11 ± 17.54* |
| 10.16 ± 1.24 | 0.50 ± 0.40 | 94.57 ± 4.55* | 10.83 ± 0.87 | 0.50 ± 0.46 | 95.06 ± 4.60* |

pellent to the German cockroach *Blattella germanica* L. In view of the fact that essential oils mainly contain monoterpenoids (Masada, 1976), Inazuka (1983) evaluated numerous monoterpenoids for repellency against *B. germanica* and found (−)-linalool, nerol, (−)-carvone, (+)-pulegol, (+)-pulegone, and (+)-isopulegol to be most effective space repellents. Among these compounds, linalool was found in the *Artemisia* essential oil and was proven to be repellent to *A. aegypti* in the present study.

Penfold and Morrison (1952) reported that of 40 Australian essential oils, the Huon pinewood (*Dacrydium franklini*) essential oil and the leaf oils of *Backhousia myrtifolia*, *Melaleuca bracteata*, and *Zieria smithii* were the most effective in repelling mosquitoes, March flies, and sand flies. Mayer (1952) reported that the essential oils of *Eucalyptus* and *Caryophyllum* repelled several species

of mosquitoes. Although these essential oils were reported to repel mosquitoes, none of the active compounds in the oils were isolated and identified.

Of the monoterpenes isolated and identified from *A. vulgaris* in this study, terpinen-4-ol and cineole were previously reported to possess repellency against mosquitoes. Thus, cyclic terpene alcohols, including terpinen-4-ol, were reported to repel mosquitoes (Lion Corp., 1982). A composition containing cineole and one or more of 2,4-diethyltoluamide, triethylene glycol monoalkyl ether, and triethylene glycol dialkyl ether was reported as a mosquito repellent for aerial and skin applications (Nitto Electric Industrial Co., 1981). ($\pm$)-Camphor, borneol, bornyl acetate, isoborneol, (+)-limonene, myrcene, $\beta$-pinene, and thujone were evaluated as repellents against *A. aegypti* (King, 1954).

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# DISPOSITION AND FATE OF CUCURBITACIN B IN FIVE SPECIES OF DIABROTICITES

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**Abstract**—Five species of diabroticites with different host-plant preferences produced an essentially identical array of metabolites when fed radiolabeled cucurbitacin B synthesized in vivo and purified from *Cucurbita maxima* Duchesne seedlings. All species excreted the bulk of the cucurbitacin (67.17–94.59% total dpm), permanently sequestered a small proportion of a cucurbitacin conjugate in the hemolymph (0.98–2.76%), and apportioned the remainder between the gut, the body, and the eggs (when present). Markedly different ratios between the excretory metabolites (i.e., polar vs. unmetabolized cuc) suggest that *Dibrotica virgifera virgifera,* a grass specialist, and *Acalymma vittatum,* a cucurbit specialist, have lower rates of metabolic alteration than the polyphagous *D. undecimpunctata howardi, D. balteata,* and *D. cristata,* which is associated with relict prairies. Mean life-spans of *D. balteata* and *D. v. virgifera* and male *A. vittatum* decreased significantly with continuous feeding on *Cucurbita* fruit containing cucurbitacins (vs. fruit devoid of cucs). The longevity of female *A. vittatum,* however, was unaffected by the presence of cucurbitacins.

**Key Words**—Cucurbitacins, *Diabrotica* spp., *Acalymma vittatum,* Coleoptera, Chrysomelidae, kairomone metabolism, detoxification.

## INTRODUCTION

A profound coevolutionary relationship exists between the diabroticite rootworm beetles (Coleoptera: Chrysomelidae: Galerucinae: Luperini) and the plants of the Cucurbitaceae. The Cucurbitaceae are remarkable for the production of relatively large quantities of a series of oxygenated tetracyclic triterpenoids, the cucurbitacins (cucs) (Lavie and Glottner, 1971; Rehm, 1960) (Figure 1). Intensely bitter substances, cucs are highly toxic to laboratory animals and ver-



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FIG. 1. Chemical structure of cucurbitacin B. Structures of other cucurbitacins are: D, 25,OH; E, 1,2 C = 1C; I, 25,OH and 1,2 C = C.

tebrate herbivores (David and Vallance, 1955; Metcalf et al., 1980; Watt and Breyer-Brandwijk, 1962). suggesting that they function to protect the Cucurbitaceae from herbivores (Metcalf et al., 1980).

At some point now remote in the coevolutionary process, ancestral diabroticites developed sensilla on the maxillary palpi specifically depolarized by cucurbitacins, promoting host selection and opening up a new ecological niche. These receptors persist today in all diabroticite species examined, giving them an appetite for the bitter Cucurbitaceae, even though some species have developed larval host affinities with noncucurbit plants.

While the pollen-feeding adults of all species are polyphagous, the root-feeding larvae of *Diabrotica virgifera virgifera* LeConte, the western corn rootworm (WCR), are essentially restricted to corn (with devastating results) and *D. cristata* Harris larvae are strongly associated with prairie grasses (i.e, *Andropogon* spp.) (Branson and Ortman, 1970; Branson, 1971). Larvae of *D. undecimpunctata howardi* Barber, the Southern corn rootworm (SCR), and *D. balteata* LeConte, the banded cucumber beetle (BCB), are polyphagous, attacking members of the Cucurbitaceae, Gramineae, Convolvulaceae, Fabaceae, etc. (Pitre and Kantack, 1962; Smith, 1966). The larvae of *Acalymma vittatum*, the striped cucumber beetle (SCB), are monophagous on cucurbits, however (Houser and Balduf, 1925).

The ability of diabroticites to feed and live upon bitter Cucurbitaceae containing as much as 0.32% fresh weight cucs (Metcalf et al., 1982) indicates the development of effective mechanisms for disposition of these toxic triterpenoids. In the following study, the metabolism of radiolabeled cucurbitacin B was examined in five species of diabroticites with different larval host specificities to ascertain the mechanisms by which detoxification is accomplished and some of its ecological consequences.

## METHODS AND MATERIALS

*Preparation of Radiolabeled Cucurbitacin B and Feeding to Beetles.* Radiolabeled cucurbitacin B was synthesized in vivo in seedlings of *Cucurbita maxima* Duchesne cv. "Mammoth Gold" from the sodium salt of DL-[2-$^{14}$C]mevalonic acid (39.5 mCi/mmol, Research Products International Mount Prospect, Illinois). Newly emerged seedlings were placed in distilled water containing the radiolabeled mevalonate and incubated at room temperature until the cotyledons were fully expanded. Seedlings were then harvested, ground in a blender in a minimal amount of water, extracted with a 10-fold excess of chloroform, and concentrated. [$^{14}$C]Cucurbitacin B was purified by thin-layer chromatography (TLC) (2×) with two different solvent systems: $CHCl_3$–MeOH (95:5); and ether–hexane–MeOH (70:30:5) (Metcalf et al., 1982). A small aliquot of purified [$^{14}$C]cuc B was quantified by UV spectrophotometry and counted by liquid scintillation to determine specific activity (3.0–5.5 $\mu$Ci/g). Approximately 1 mg of the purified [$^{14}$C]cucurbitacin B was then placed on a small piece of silica gel TLC plate, or, in some instances, on a small piece of squash fruit (*C. moschata* cv. "Waltham Butternut") to facilitate ingestion of the labeled cuc by the smaller beetle species reluctant to feed on silica gel.

All beetles were obtained from laboratory cultures except for *D. cristata* which was collected locally. After 24 hr of starvation, twenty 1- to 2-week-old beetles (*D. cristata* age unknown) were placed in a glass 25-ml Ehrlenmeyer flask with the [$^{14}$C]cucurbitacin B and a cotton wick soaked in water. As a result of the low specific activity and consequently large amount of cuc B necessary to detect the metabolites, beetles were fed ad libitum for 48 hr. All species except *A. vittatum* had consumed all of the labeled cucurbitacin at that time.

At 48 hr, hemolymph was collected by cervical puncture and applied directly to a 0.25-mm silica gel TLC plate with fluorescent indicator (F254). The gut was carefully dissected and the excreta collected. The three fractions (gut, body minus gut, excreta) were then separately ground, extracted with methanol (3×), centrifuged, concentrated under $N_2$, and applied to the silica gel TLC plate. Oxidation of tissue residues and measurement of $^{14}CO_2$ showed that 100% of the dpms from the gut and eggs were extracted, 98% from the excreta and 88% from the body. After development of the TLC plate in chloroform–methanol (95:5), autoradiography was performed. Compounds that differentially affected the mobility of cucurbitacin B and its metabolites were present in the various extracts so that $R_f$ values differed slightly between the extracts. Areas of the TLC plate corresponding to spots on the autoradiograph were scraped and counted by liquid scintillation. To determine the number of compounds located at the origin, a small portion of this spot ($R_f$ = 0.00) was counted but the bulk rechromatographed (TLC) in pure methanol, followed by autoradiography.

To better ascertain the long-term equilibrium of cucurbitacin and its metabolites in *D. u. howardi,* a second experiment was run. In this instance, 20 SCR were fed 7 mg of cucs B and D combined. When all cucs were consumed (72 hr), the beetles were placed on an artificial pollen diet for two weeks prior to dissection.

*Cucurbitacin Bioassy.* The presence of biologically active cucurbitacins was routinely verified by beetle feeding on TLC plates, aptly dubbed "beetleprints" (Metcalf et al., 1982). Samples were applied to 0.10-mm silica gel plates with fluorescent indicator and plastic backing. The developed plates were then placed in a cage with ca. 100–200 adult *D. u. howardi* or *D. v. virgifera.* The beetles ingested the areas of the plate containing the cucurbitacins, leaving a permanent record both qualitative (comparison of $R_f$ values to standards) and semiquantitative (size of area eaten). As *D. u. howardi* can detect as little as 1 ng of cucurbitacin B, this is an extraordinary sensitive and effective bioassay (Metcalf et al., 1980).

*Purification and Identification of Hemolymph Cucurbitacin.* Pooled hemolymph samples were removed by cervical puncture from cucurbitacin-fed *D. u. howardi* and applied directly to 0.25 mm silica gel TLC plates with fluorescent indicator. After initial purification by TLC ($CHCl_3$–MeOH, 1 : 1, $R_f = 0.40$), the hemolymph cucurbitacin was further purified by HPLC (Waters Assoc., Milford, Massachusetts model 510; C18 column; solvent, 73% methanol; flow rate, 1.0 ml/min; UV detector at 210 nm). Of the ten peaks resolved, three peaks comprised 80% of the total sample. Four fractions were collected, but only fraction 3 elicited avid feeding by *D. u. howardi* beetles. Two major peaks in this fraction were further resolved by HPLC but only one peak ($R_t$ = 4.26 min) elicited strong beetle feeding. Prior to high-pressure liquid chromatography, this peak comprised 21% of the total sample but was purified by HPLC to 94.6% of the total sample. This sample was then submitted in thioglycerol for positive-ion fast atom bombardment mass spectrometry on the ZAB-HF mass spectrometer located at the University of Illinois (Rhinehart, 1982).

The TLC-purified cucurbitacin conjugate was incubated overnight with pectinase (Sigma, P2401) in 0.2 M sodium phosphate buffer (pH 4.5) to free the cucurbitacin which was then extracted with chloroform and submitted for 70-eV direct-probe electron impact (EI) mass spectrometry. Pectinase proved most suitable for hydrolyzing the cuc conjugate despite its being a very crude enzyme containing many contaminants which could be the "active" enzyme. After treatment with pectinase and thin-layer chromatography, the TLC plates were sprayed with 5% ferric chloride in ethanol (w/v) to distinguish the cucs with a diosphenol group in ring A (cucs E and I) that produce a violet color from those that show no reaction (cucs B and D) (Metcalf et al., 1980).

*Quantification of Cucurbitacins in Hemolymph. D. balteata* adults fed ad libitum on cucurbitacin-containing *C. andreana* × *C. maxima* fruit (Rhodes et

al., 1980) were removed at given intervals and hemolymph collected by cervical puncture from five beetles on each sampling date. The pooled blood samples (3–7 $\mu$l) were placed directly on a 0.25-mm silica gel TLC plate with fluorescent indicator. After development in chloroform–methanol (1 : 1), the quenched areas ($R_f$ = 0.40) were scraped, eluted in methanol, and the cucurbitacin measured by UV spectrophotometry at 210 nm (Metcalf et al., 1980). Cucurbitacin was quantified using a standard curve of pure cuc E glycoside prepared as described by Enslin (1954).

*Longevity Curves.* Newly emerged adults of each species were randomly placed in groups of 50 in two Plexiglas cages (30 cm height × 15 cm diameter). The two groups received either sweet squash fruit devoid of cucs (*Cucurbita pepo* cv. "White Bush Scallop," "Summer Yellow Crookneck," or *C. moschata* cv. "Waltham Butternut") or bitter squash (*C. andreana* × *C. maxima* $F_1$ hybrid) containing 1–2 mg cucs B and D per gram fresh weight. These uniformly sized pieces (1 cm × 2.3 cm diameter) weighed about 3 g each and were replenished as necessary (approximately every 2–4 days). The cages were observed daily and dead beetles removed promptly. Total cucurbitacin consumption per beetle was estimated by knowledge of the number of beetles, the number of disks consumed over a given time period and cucs per gram of fruit, and assuming one fourth of each disk was consumed. The median longevity (days) was selected for the end point in time for calculations to make the prior mentioned assumption more plausible (e.g., one fourth of the disk would not be consumed if only one beetle were alive.)

## RESULTS

All species produced an essentially identical array of major cucurbitacin B metabolites in the excreta, body, gut, and hemolymph as evident in Table 1. Although all species excreted the bulk of the label (67.17% for SCB to 94.59% for BCB), *A. vittatum* and *D. v. virgifera* excreted a larger proportion as the presumably unmetabolized cucurbitacin ($R_f$ = 0.53). Bioassay of the excreta for compounds with feeding-stimulant activity consistently revealed only one compound ($R_f$ = 0.53) capable of eliciting a feeding response from the beetles (Figure 2E). The remarkable sensitivity of diabroticites to cucurbitacins (as low as 1 ng) dissuades one from implicating insufficient quantities of the major excretory metabolites as the cause for lack of feeding (Metcalf et al., 1980).

In contrast to *D. v. virgifera* and *A. vittatum*, however, *D. balteata, D. cristata,* and *D. u. howardi* metabolize the majority of cuc B to more polar end products (i.e., $R_f$ = 0.00, 0.15, 0.23) prior to excretion. Rechromatography (TLC) of the spot at the origin followed by autoradiography demonstrated two spots in the excreta of *D. cristata* ($R_f$ = 0.00, 0.47) but only one spot ($R_f$ =

TABLE 1. QUANTITATIVE RELATIONSHIPS AMONG EXTRACTABLE [$^{14}$C] CUCURBITACIN B METABOLITES IN FIVE SPECIES OF DIABROTICITES

| | | Dpm as % of species total dpm | | | | |
|---|---|---|---|---|---|---|
| Sample | $R_f^a$ | *D. u. howardi* | *D. balteata* | *D. virgifera* | *D. cristata* | *A. vittatum* |
| Excreta | 0.00 | 40.62 | 31.35 | 27.35 | 43.81 | 24.70 |
| | 0.15 | 24.33 | 45.70 | 9.20 | 20.38 | 12.38 |
| | 0.23 | 16.47 | 13.98 | 10.28 | 7.14 | 12.02 |
| | 0.29 | 1.54 | | | | |
| | 0.47 | 0.26 | | | 2.01 | |
| | 0.53 | 1.72 | 3.56 | 29.83 | 7.12 | 18.07 |
| Total | | 84.92 | 94.59 | 76.66 | 81.00 | 67.17 |
| Body | 0.00 | 4.34 | 0.75 | 14.59 | 6.85 | 8.76 |
| | 0.15 | 1.62 | 0.48 | 5.29 | 0.83 | 0.93 |
| | 0.22 | 1.09 | 0.11 | | 0.34 | |
| | 0.29 | 0.38 | | | | |
| | 0.50 | 1.42 | | 0.51 | 0.32 | 11.80 |
| Total | | 8.85 | 1.34 | 20.39 | 8.02 | 21.49 |
| Gut | 0.00 | 2.70 | 2.21 | 1.12 | 8.31 | 4.98 |
| | 0.16 | 1.34 | 1.11 | | | |
| | 0.22 | 0.99 | 0.75 | | | |
| | 0.49 | 0.17 | | | | 3.62 |
| | 0.66 | 0.03 | | | | |
| Total | | 5.23 | 4.07 | 1.12 | 8.31 | 8.60 |
| Hemolymph | 0.00 | 0.89 | | | 2.35 | 1.33 |
| | 0.53 | 0.09 | | | | 1.43 |
| Total | | 0.98 | 0.00 | 0.00 | 2.35 | 2.76 |

[a] Methanol extracts (excreta, body, gut) and hemolymph applied to 0.25-mm silica gel thin-layer plate; solvent chloroform–methanol (95:5); cucurbitacin B standard $R_f$ = 0.51; cucurbitacin D standard $R_f$ = 0.43.

0.17) in *D. v. virgifera*. Thus, the more polar metabolites of cuc B in the excreta may not be identical in all of the species.

Widely varying amounts of the label were found in the body extracts of the different species. *A. vittatum* contained 21.49% of the ingested cuc in the body, with more than 50% of this present as the unmetabolized cuc. *D. v. virgifera,* again rather unique among the *Diabrotica* spp., retained 20.39%, mostly of the more polar cuc metabolites. The more polar cuc metabolites predominated in the gut extracts of all species, except for *A. vittatum* which had more than one third present as the unmetabolized cucurbitacin.

The more polar cuc metabolites again predominated in hemolymph extracts of *D. u. howardi* and *D. cristata,* but *A. vittatum* showed an equivalent amount of the apparently unmetabolized cuc. Due to inability to obtain sufficient hemolymph, no label was detected in *D. balteata* or *D. v. virgifera,* but subsequent

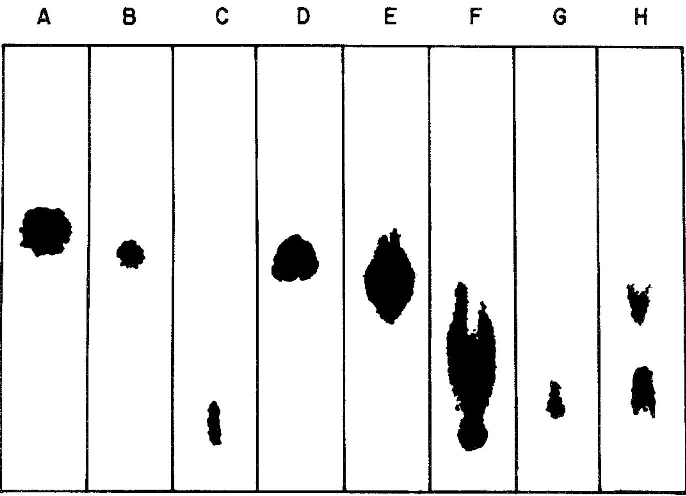


FIG. 2. Profiles of thin-layer chromatograms with cucurbitacin-containing areas eaten by diabroticites shown in black. (A, B) cucurbitacin B and D standards, respectively; (C) 0.75 $\mu$l hemolymph from field-collected SCR; (D) SCR hemolymph incubated with pectinase prior to TLC; (E) excreta from SCR fed cuc-containing fruit; (F) body of SCR with no exposure to cucs for 6 weeks; (G) 200 eggs from SCR adults fed cucs; (H) 400 eggs from SCB adults exposed to cucs only as larvae.

experiments with unlabeled cucs revealed the presence of only one cuc metabolite in all four *Diabrotica* spp. (Figure 2C). As Figure 3 demonstrates, the cuc metabolite rapidly accumulates in the hemolymph of adults fed cucurbitacin-containing fruit. SCB larvae reared on *C. maxima* cv. "Blue Hubbard" roots containing cucurbitacins even retain sufficient quantities in the hemolymph through pupation to render the newly emerged adults extraordinarily bitter tasting (Ferguson and Metcalf, 1985). Rather large amounts of cuc metabolites, perhaps originating from the hemolymph, are found in the eggs of SCB, SCR, and BCB (Figure 2G and H). Even SCB larvae reared on *Cucurbita moschata* cv. "Waltham Butternut," which has trace amounts of cucs located only in the seedling roots (Ferguson, unpublished data), deposited eggs with surprising amounts of cucs. The cucurbitacins, however, may be in the sticky mucous accompanying the eggs and not within the eggs proper.

SCR fed radiolabeled cucs B and D followed by two weeks on an artificial pollen diet prior to dissection revealed a tissue distribution of cuc metabolites analogous to that seen in Table 1 for SCR. A smaller proportion was located in the gut (0.20%) and body (4.54%) with a concomitantly larger amount in the excreta (93.93%), suggesting that a portion of the gut and body labeled compounds in the 48-hr experiments were transient, e.g., being metabolized prior to excretion. The hemolymph retained 1.12% of the total dpm, and the 1058 eggs collected during the two week period contained 0.21%.

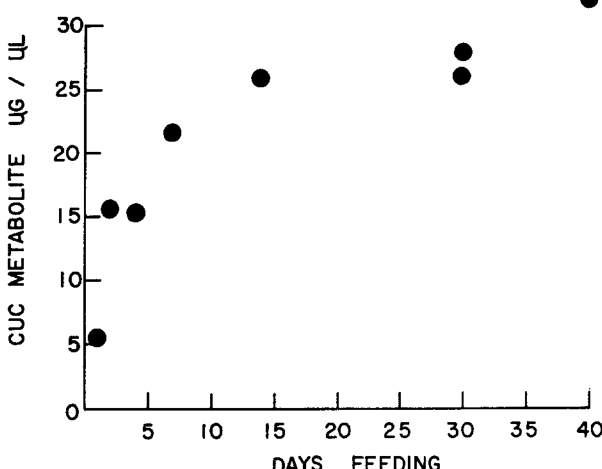


FIG. 3. Accumulation of cucurbitacin conjugate in the hemolymph of *D. balteata* with continuous feeding on bitter fruit of the $F_1$ hybrid *Cucurbita andreana* × *C. maxima* cv. "Mammoth Gold."

The $R_f$ values of some excretory and body metabolites from cucs B and D metabolism were unique from those of cuc B metabolites only. Thus, deacetylation (cuc B → cuc D) is probably not one of the initial steps for excretory or body cucurbitacin derivatives.

*Effects of Consumption of Bitter Squash upon Diabroticite Longevity.* The paired mortality patterns for adult beetles fed on sweet or bitter squash are shown in Figure 4. This technique has been shown to be a sensitive indicator of the effects of dietary xenobiotics on insects (Lu et al., 1978). For the two *Diabrotica* species tested (BCB, WCR), the mean life-span for the adults fed bitter fruit was significantly shorter than those fed sweet fruit ($t$ test, $P < 0.001$). For *A. vittatum* significant differences in mean life-span were detected for male but not for female beetles ($t$ tests, ♂ $P = 0.018$, ♀ $P = 0.668$). Significant decreases in mean longevity were also evident in male and female *D. v. virgifera* with continuous feeding on bitter fruit, yet mean male life-span decreased 147% while mean female life-span decreased only 39%. This compares to a 27% decrease for male *A. vittatum*. Preliminary evidence suggests male diabroticite feeding is stimulated to a greater extent than female feeding by equivalent amounts of cucurbitacin incorporated into an artificial pollen diet (Ferguson, unpublished data). Thus, the relatively greater reduction in male longevity with continuous feeding on bitter fruit may merely reflect increased ingestion of the toxic cucurbitacins. Nutritional deficiencies of the bitter fruit when compared with the sweet fruit cannot be positively excluded as the cause of the shortened life-span, however.

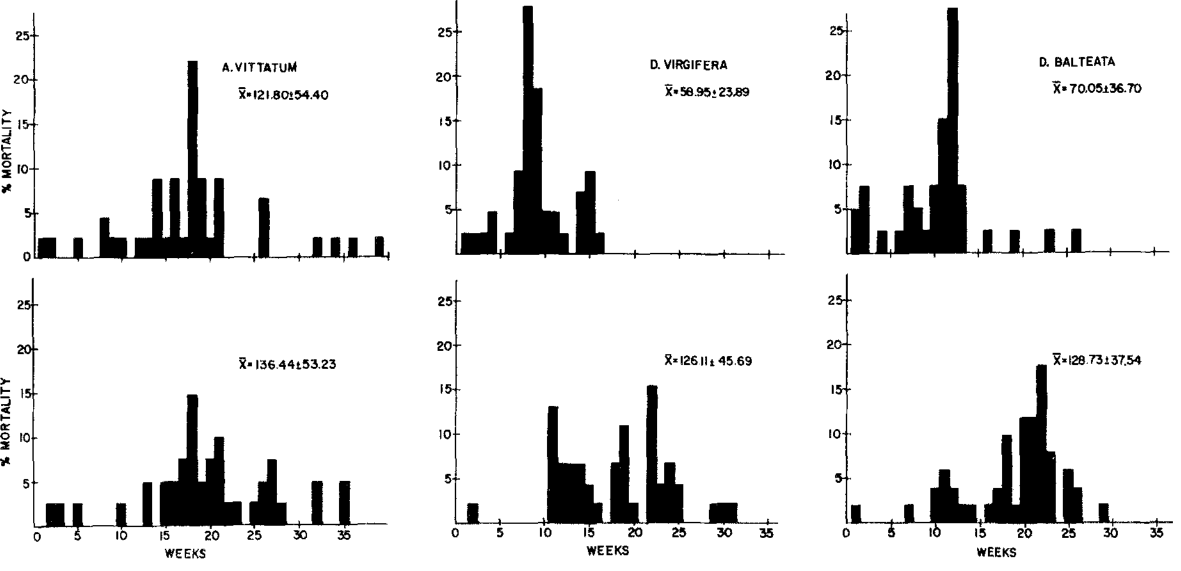


FIG. 4. Weekly mortality patterns of lab-reared *Acalymma vittatum*, *Diabrotica virgifera virgifera*, and *D. balteata* adults continuously fed sweet *Cucurbita* fruit with no cucurbitacins (lower row) and bitter *Cucurbita* fruit with cucurbitacins B and D (upper row).

Over their lifetimes, adult diabroticites supplied with cucurbitacin-containing fruit consumed an estimated 1.0–2.0 mg cucs B and D/BCB beetle, 0.8–1.6 mg/WCR, and 1.7–3.4 mg/SCB beetle. For SCB, this translates into an impressive 139–277 mg cucs/g body weight, which is astonishing in light of the recorded mouse $LD_{50}$ (intraperitoneal) for cuc B of 0.03 mg/g body weight (David and Vallance, 1955). Mean male life-spans were significantly longer than female life-spans on fruit without cucurbitacins.

*Characterization of Hemolymph Cucurbitacin.* The TLC-purified cucurbitacin freed by pectinase treatment had a retention time identical to an authentic cuc D standard (mp = 151–153°C) on HPLC ($R_t$ = 5.60) and cochromatographed (TLC) with cuc D as demonstrated by fluorescence quenching and beetleprints (Figure 2B and D) ($R_f$ = 0.46, $CHCl_3$–MeOh, 95:5). Cucurbitacins D and I are $C_{25}$-desacetoxy cucs B and E, respectively (Figure 1). Mass spectrometry showed the cuc to also have a fragmentation pattern identical to an authentic cuc D standard (Figure 5) (Audier and Das, 1966). Analogously, beetles fed fruit containing cuc E and cuc E glycoside released cuc I ($R_f$ = 0.52; $FeCl_3$ = +) when the hemolymph cucurbitacin was treated with pectinase. Neither cuc E or cuc B standards when incubated with pectinase yielded any change in $R_f$ value by TLC. Thus, the beetles and not the pectinase were removing the acetoxy groups. The hemolymph moieties then are almost certainly derived via deacetylation followed by conjugation, in contrast to the excretory cuc metabolites.

Two individually purified samples of the hemolymph cucurbitacin moiety,

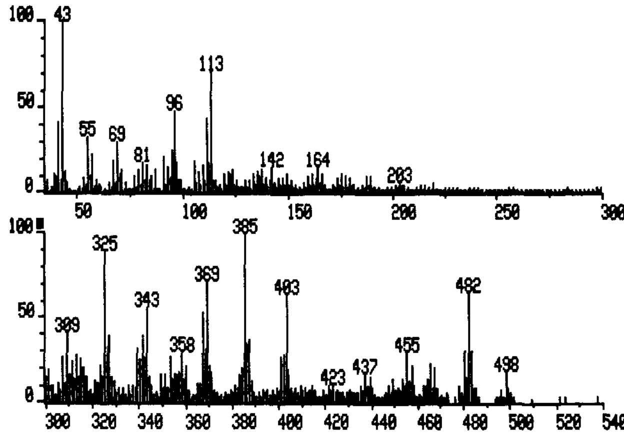


FIG. 5. Electron impact mass spectrum of cucurbitacin resulting from pectinase hydrolysis of cuc conjugate purified from the hemolymph of *Diabrotica undecimpunctata howardi* beetles. Spectrum is consistent with cucurbitacin D, $C_{30}H_{44}O_7$, mol wt = 516; *m/e* 498 ($M^+ - H_2O$), 403 (ring structure-side chain), 385 (403 $-H_2O$), 369 ($C_{24}H_{33}O_3$), and 96 ($C_8H_8O$ side chain).

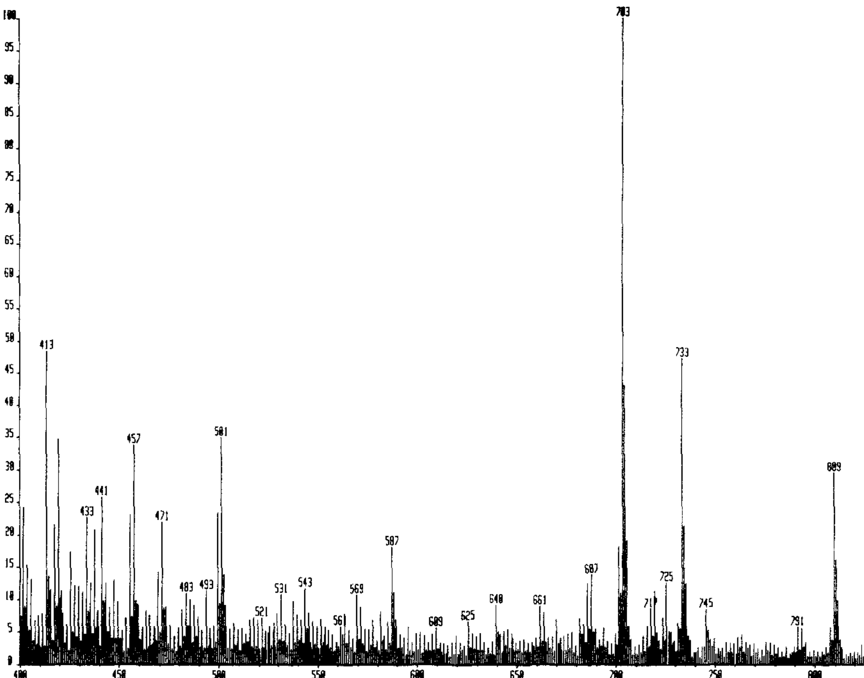

FIG. 6. Positive-ion fast-atom bombardment mass spectrum of the cucurbitacin metabolite purified from the hemolymph of *Diabrotica undecimpunctata howardi*.

submitted for fast atom bombardment (FAB) mass spectrometry, yielded essentially identical spectra with a molecular ion of 809 $M^+$ (Figure 6). The spectrum is suggestive of glutathione being the conjugated moiety, but only further chemical characterization of the hemolymph conjugate that is now in progress can substantiate this preliminary interpretation.

## DISCUSSION

Due to the very low specific activity of the purified radiolabeled cucurbitacin B, each beetle consumed an unusually large amount of cuc B ($\approx$ 50 $\mu$g/beetle) compared to the prospects for short-term ingestion in the wild. This excessive load of xenobiotic could theoretically stress the beetles' detoxification system and produce an atypical metabolic fingerprint. Yet, even when the beetles were fed 350 $\mu$g/beetle, an essentially identical metabolite pattern was discernible, and no evidence to date has revealed any deleterious short-term effects of cucurbitacins upon diabroticites. The production of the cucurbitacin metabolites by gut microorganisms cannot be positively excluded (Brand et al., 1975), yet organisms producing identical metabolites would necessarily be present in all diabroticite species examined.

To facilitate excretion, metabolic detoxification systems commonly increase the water solubility (polarity) of an ingested xenobiotic by introducing a hydrophilic function via oxidation, hydrolysis, reduction, and/or conjugation with a polar endogenous moiety, e.g., glucose, glutathione, amino acids, sulfate moieties, etc. (Dauterman and Hodgson, 1978). With one exception ($R_f = 0.66$ in the SCR gut), all cucurbitacin B metabolites were more polar than cuc B itself. None of these more polar cuc products should be positively equated with "detoxified" cuc B products without toxicity testing, however. In order to cope with the vast diversity of xenobiotics abroad in the insect habitat, insect detoxification enzymes are generally nonspecific. Consequently, more toxic metabolites are produced occasionally, e.g., sinigrin conversion to allylisothiocyanate (Blum, 1983).

None of the excretory metabolites of cucurbitacin B ever elicited a feeding response from the beetles or were discernible by quenching under UV light except the apparently unmetabolized cucurbitacin B. The destruction or loss of the appropriate physical conformation to trigger the diabroticite cuc receptor could, quite ironically, correspond to metabolic detoxification. Metcalf et al. (1980) have suggested that the appropriate cuc triggering conformation is associated with the orientation of the oxygens on the cucurbitacin molecule, propitious targets for detoxification enzymes.

In the few comparative studies examining the metabolism of plant secondary compounds in insects [excepting the idiosyncratic process of sequestration (Blum, 1983)], unique metabolic pathways have generally not distinguished adapted (specialist) species from nonadapted or generalist species (Bull et al., 1984; Self et al., 1964). Rather greater toxicity of some plant toxins to nonadapted species has been associated with greater body burdens of the unmetabolized toxin along with lower excretion rates. For example, immature stages of three tobacco feeding insects (tobacco wireworm, cigarette bettle, differential grasshopper) and adult houseflies all metabolized 70–79% of a dermal dose of nicotine to the nontoxic cotinine, but the latter was singularly unable to excrete 90% of the administered dose at 18 hr (Self et al., 1964). On the other hand, some lepidopterous tobacco feeders, the tobacco budworm, and the cabbage looper excrete nicotine with no apparent metabolic changes.

Regardless of their current host-plant associations, all diabroticite species produced an essentially identical array of major metabolites from cuc B. Distinctly different ratios between the metabolities were often discernible, however, suggesting different rates of metabolism. The polyphagous SCR and BCB produced the largest proportion of polar excreta products, followed by WCR and *D. cristata,* associated with the Poaceae, and finally, SCB which are monophagous on cucurbits. The high proportion of the apparently unmetabolized cuc B in the excretory products of SCB and WCR may reflect absorption and excretion via the Malpighian tubules without metabolic alteration.

In conjunction with the greater excretion of the unmetabolized cuc B in

SCB and WCR, the apparently greater body absorption of cuc B and its metabolites suggests a lower rate of metabolic alteration. Indeed, Chio and Metcalf (1979) found the order of effective detoxification for four insecticides to be, in general, SCB < WCR < SCR. A portion of the label in the body may represent hemolymph contamination, however, as complete exsanguination proved impossible. Additionally, a portion of the body or gut label may be transient, e.g., being metabolized prior to excretion.

The successful excretion of a xenobiotic without an energy input for "detoxification" would undoubtedly be highly advantageous, particularly to a highly adapted specialist like *A. vittatum*. Clearly, female *A. vittatum* dispose of cuc B. effectively enough to remain unscathed by the toxic cucurbitacins despite greater body burdens and a lower rate of metabolic alteration. Furthermore, female SCB showed no adverse effects from the ingestion of bitter fruit even though they were exposed to (and assuredly sequestered) cucurbitacins from their larval host *C. maxima* cv. "Blue Hubbard." As male and female longevity are not significantly different on bitter fruit, the 25% decrease in male longevity may be inconsequential in the wild. Evaluation of five *Cucurbita* spp. cultivars with known cucurbitacin content (Ferguson, unpublished data) for the rearing of SCB, show the three cucurbitacin-containing cultivars to be superior (Reed et al., 1984). All SCB collected from the field contained cucurbitacin in their hemolymph, but only 10–20% of the *Diabrotica* species did (Ferguson and Metcalf, 1985).

In conjunction with its significantly shorter life-span upon the ingestion of bitter fruit (and by inference cucurbitacins), the apparently decreased ability by *D. v. virgifera* to metabolize and subsequently excrete cucurbitacin B could figure prominently in its current host affinities. Reductions in fecundity and lower rates of increase may or may not be secondary consequences of decreased longevity. Observations from laboratory rearing of *Diabrotica* spp. suggest that the presence of cucurbitacins inhibits oviposition in mated adults. In addition, cucurbitacins appear to inhibit orientation of male *D. u. howardi* to pheromone traps (Andersen, 1981). Thus, *Diabrotica* spp. (polyphagous and monophagous) seemingly experience multiple adverse effects associated with the ingestion of cucurbitacins which *A. vittatum* does not.

Ecological benefits do accrue to all the diabroticites from the consumption of these toxic compounds, however. The presence in the hemolymph of the cucurbitacin metabolite renders the beetles unpalatable to potential predators and, in addition, could potentially protect them from internal parasites, etc. (Ferguson and Metcalf, 1985). The eggs contain significant amounts of biologically active cucurbitacin following adult, or in some instances, larval ingestion of cucs. The egg cuc may originate from the hemolymph, as adults long removed from a cuc source still deposit eggs containing cucs. Thus, cucs may be effective defense compounds throughout the life cycle of the diabroticites.

The rapid accumulation of the hemolymph cuc (Figure 4) would yield rel-

atively immediate effects via à vis predators and parasites, clearly advantageous to the beetles, while the reduction in life-span would be realized more slowly. From all evidence the hemolymph cucurbitacin is permanently sequestered, and whether it is, in itself, toxic to the beetles is uncertain. Most importantly, rarely would a diabroticite in the field consume only bitter squash over its life-span, except perhaps *A. vittatum,* which is monophagous on cucurbits, and female SCB remain unscathed by the consumption of large amounts of cucurbitacins. Consequently, deleterious effects on longevity in the field may be relatively insignificant and associated only with extensive feeding periods, unlikely occurrences for the polyphagous *D. balteata, D. undecimpunctata howardi,* and the Poaceae specialists *D. v. virgifera* and *D. cristata.*

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# STIMULATORY EFFECTS OF TANNINS AND CHOLIC ACID ON TRYPTIC HYDROLYSIS OF PROTEINS: ECOLOGICAL IMPLICATIONS

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**Abstract**—The biochemical basis for considering tannins as digestion inhibitors has been reexamined. Both stimulatory and inhibitory effects of tannins on tryptic hydrolysis are reported. We show how stimulation may result from tannin-induced structural changes in the substrate protein, effectively denaturing it. The surfactant and bile constituent cholic acid also produced similar stimulatory effects. These results have considerable implications for normal digestive physiology as well as for the impact of tannins on argicultural and natural herbivore populations.

**Key Words**—Tannins, digestion, herbivory, digestibility-reducing substances, gut surfactants, bile acids, tannin-protein interactions.

## INTRODUCTION

The notion that tannins provide a quantitative defense against herbivory has been a central theme in the development of plant defense theory (Rhodes and Cates, 1976; Feeny, 1976). Their allelochemic activity is considered to arise primarily from the formation of insoluble tannin–protein complexes during the mastication of food, thus denying substrate to proteolytic enzymes in the gut (Feeny, 1969). It has also been suggested (Swain, 1979) that they can act directly on gut enzymes in an analogous manner and tan the cells of the gut wall so as to block the passage of nutrients from the gut. However, in a recent review, Bernays (1981) has pointed out several instances where tannins show no adverse effects on insect herbivores, and she has shown that they can even act as phagostimulants. Feeny (1969) proposed that the allelochemic effects of tannins could be moderated by the high pH of the midgut found in some insects, this



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causing at least partial dissociation of insoluble complexes and allowing the protein to become more freely available for digestion. Following this, Berenbaum (1980) has shown that high midgut pH is correlated with phytophagy in the Lepidoptera, although House (1974) had earlier argued that over a range of taxa this correlation is unsatisfactory and no better than that for taxonomic variation. Despite its ecological importance, the basic biochemical evidence to support the hypothesis that a high midgut pH is of adaptive significance does not appear to have been explored experimentally since the initial paper by Feeny (Zucker, 1983).

Soluble tannin–protein complexes have been identified (Calderon et al., 1968), indicating that dissolving an insoluble complex by raising the pH need not eliminate interaction between tannin and protein. In this paper we show that under certain conditions interactions between tannins and proteins can generate soluble complexes, and we demonstrate the occurrence of inhibitory, stimulatory, and null effects on the tryptic proteolysis of these complexes. We have selected the protease trypsin, as it is a very important enzyme in mammalian herbivores and as the occurrence of a "trypsin-like" enzyme in insects is also well documented (House, 1974). In the absence of information for herbivorous insect species, the trypsin concentration used in our experiments is in the range reported for simuliids (House, 1974). Ecologically valid tannin–protein (T/P) ratios have been employed, with particular attention being paid to conditions that enhance proteolysis.

## METHODS AND MATERIALS

The tryptic digestion of bovine serum albumin (BSA, Sigma fraction V; trypsin, Sigma type II) was studied in the presence of various concentrations of tannin at 37°C. Two tannins were used in separate series of experiments: tannic acid (BDH Chemicals Limited) and quebracho tannin (Harshaw Chemicals, Glasgow), the influence of both tannins being studied in each proteolytic system described below. The tannic acid represents a relatively pure example of a hydrolyzable tannin, while the quebracho tannin is a mixture of phenolic compounds in which condensed tannins predominate.

Proteolytic digestion of the BSA or BSA–tannin complex was carried out in a total volume of 25 ml containing 50 mM citrate–phosphate buffer ($Na_2HPO_4$ adjusted to pH 7.5 with citric acid), BSA at 4 mg/ml, and trypsin at 200 units/ml. Control reactions (tannin-free) and reactions with tannin–protein weight ratios of 0.05 to 2.0 were used. Solutions of tannin and protein were made separately and mixed 15 min before the start of the experiments to ensure time for full complexation. At zero time, trypsin (in 5 ml of 0.001 M HCl) was added to the tannin–protein mixture, making it up to its final volume of 25 ml. After 1 min and at timed intervals for the following 2 h, 1-ml portions were removed

and added to 2 ml 5% trichloracetic acid (TCA) which precipitated the undigested protein. Portions (250 $\mu$l) of the supernatant TCA solution were added to 500-$\mu$l aliquots of ninhydrin reagent (Sigma reagent, as described in Moore and Stein 1968). Procedure then followed Moore and Stein (1954) with the ninhydrin reaction being incubated for 15 min at 100°C, cooled, diluted with 50% EtOH, and the absorbance due to TCA-soluble proteolysis products measured at 570 nm. Preliminary experimentation showed that tannins did not interfere with the assay. Experiments were performed in triplicate with at least one control per batch. The pH of reaction mixtures was checked at the end of each run to confirm that no change had occurred.

The procedure used above was modified in further proteolysis experiments as follows. In experiments to examine the influence of heat denaturation on tannin-free proteolysis, the protein solution used in the experiment was heated to 90°C for 1 min and then allowed to cool to room temperature before use.

Two proteins other than BSA were used in parallel lines of experiments, these being $\gamma$-globulins (Sigma, bovine, Cohn fraction II) and hemoglobin (Sigma, human). In these experiments, the buffer pH was changed to 8.5 (50 mM boric acid adjusted to pH with sodium hydroxide).

In experiments on all three substrate proteins, a procedural modification was used to mimic events in mastication. In these experiments doubly concentrated unbuffered tannin and protein solutions were mixed to form precipitates which were then redisolved by the addition of an equal volume of doubly concentrated buffer to give the standard initial system. The final modification made was to repeat experiments on BSA in pH 6.2 buffer (50 mM $Na_2HPO_4$ adjusted to pH 6.2 with citric acid) where precipitation occurs in buffered conditions. Precipitates were then redissolved by the addition of cholate (cholic acid solution, Sigma) to a final concentration of 8.0 mM in the system.

## RESULTS AND DISCUSSION

The first series of experiments investigated the interference of the two tannins on the tryptic digestion of BSA at pH 7.5 where tannin–protein precipitates do not form (Hagerman and Butler, 1978). A typical set of results using quebracho tannin, a condensed tannin (Haslam, 1981), is shown in Figure 1. For T/P = 0.1, a clearly enhanced rate of proteolysis was seen at digestion times of less than 1 h, and this declined towards the reaction rate of the control at longer times. At T/P = 0.1 this pattern was also observed for tannic acid, a hydrolyzable tannin (Haslam, 1981). By contrast at T/P = 2.0, quebracho tannin showed a marked inhibition of proteolysis (Figure 1), and this was also observed at T/P = 1.0 for tannic acid. At T/P = 1.0 quebracho tannin had no effect on proteolysis; this null point was close to T/P = 0.5 for tannic acid.

It has long been known that both low-molecular-weight phenolic com-

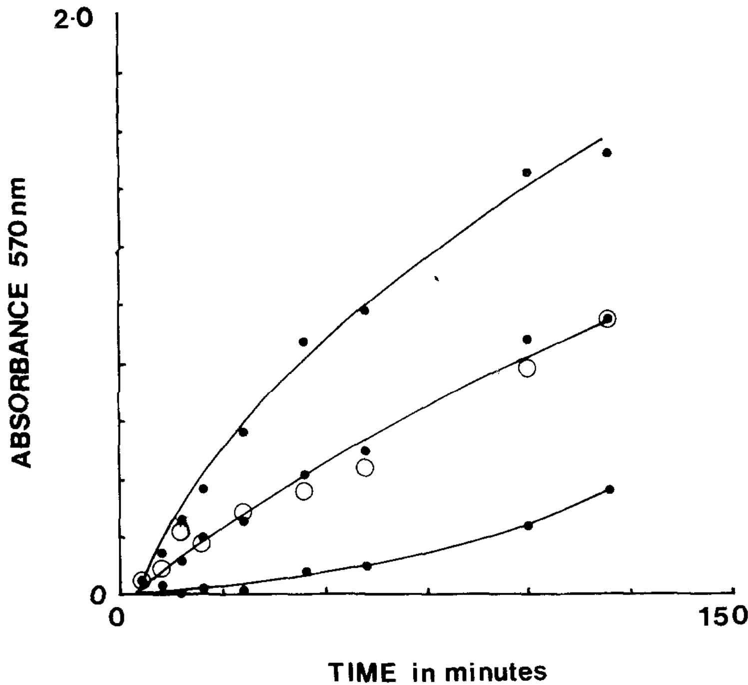


FIG. 1. Tryptic proteolysis of BSA in the presence of quebracho tannin. The upper, middle, and lower curves have tannin–protein ratios of 0.1, 1.0, and 2.0, respectively. Points marked ○ are for a tannin-free control.

pounds (Green and Neurath, 1954) and high-molecular-weight polyelectrolytes (Dellert and Stahman, 1955; Morawetz and Sage, 1955) can enhance the rate of tryptic proteolysis. Spensley and Rogers (1954) proposed that polyanions of high molecular weight with a filamentous structure would be the most effective enhancing agents, e.g., polyacrylic and polyglutamic acids. At high pH, with phenolic groups ionized, the similarity between condensed tannins (Haslam, 1981) and these structures is striking.

In the case of the polyelectrolytes, the mechanism of stimulation of proteolysis is thought not to involve trypsin directly; conformational changes in the structure of the substrate protein are believed to render the latter more readily available to the protease (Green and Neurath, 1954; Dellert and Stahman, 1955; Spensley and Rogers, 1954). This idea is supported by observations showing that tryptic autolysis is enhanced when this protein interacts with negatively charged surfaces (Johnson and Whateley, 1972). We propose that the mechanism of enhancement of BSA proteolysis noted here could also be caused by conformational changes in the structure of the protein, brought about by its interaction

with tannin. At higher T/P ratios the inhibition of proteolysis is assumed to be due to polyphenolic compounds covering the protein surface (McManus et al., 1981) leading to interference with the interaction of enzyme and substrate.

Heat-denatured BSA also showed increased rates of proteolysis compared with those of undenatured controls. However, addition of tannin to this system (T/P = 0.1) gave no further stimulation of proteolysis and, hence, results support the hypothesis that tannins induce conformational changes promoting tryptic attack.

With $\gamma$-globulin and hemoglobin, both of which have more alkaline isoelectric points than BSA, completely soluble tannin–protein systems (i.e., no precipitation or turbidity) were produced only at higher pH values, > 8.5 (Hagerman and Butler, 1978). Proteolysis experiments repeated at this pH with these proteins gave quite different results to the first series with BSA. From T/P = 0.1 to T/P = 2.0, there was no observable stimulation or inhibition, and we conclude that there is no complex formation between tannin and substrate or enzyme at this pH using these conditions.

The binding of tannins to substrate protein appeared to cause the observed changes in proteolysis, although the possibility that the effect was due to indirect interaction of tannin with "third-party" intermediates (e.g., cations influencing protein conformation) cannot be ruled out. The initial procedure was then changed to that where precipitates were formed in unbuffered aqueous solutions, as would be expected to occur during mastication, and then redissolved. This procedure gave increased rates of digestion of both $\gamma$-globulin and hemoglobin (Table 1). However stimulation of proteolysis was found at even higher values of T/P and, moreover, this was greater than at low values of T/P where there was either less stimulation or no observable effect.

When this more realistic procedure was applied to BSA at pH 7.5 and 8.5, inhibition of proteolysis was found at all values of T/P from 0.1 to 2.0. Thus, as with the other two proteins, there appears to be a difference in the tannin–protein interactions in the soluble and precipitated complex. In the formation of insoluble tannin–protein complexes, there may well be stronger interaction between tannin and protein than in cases where no precipitation occurs. BSA is reported (Hagerman and Butler, 1981) to have a relatively high affinity for tannins, certainly much greater than that of either $\gamma$-globulins or hemoglobin. If, because of this stronger interaction, some of the BSA becomes so tightly bound that it is unavailable to trypsin after dissolution, this could have the effect of masking the enhanced proteolysis occurring in that portion of the BSA that remains available. This finding illustrates the complexity of any consideration of tannin–protein interactions in protein nutrition; clearly different results can be obtained by using the same substrates and different complexation conditions. The difference between BSA and the other two proteins may simply be due to their different affinities for the tannin and the experimental conditions selected.

TABLE 1. TRYPTIC HYDROLSIS OF VARIOUS SUBSTRATE PROTEINS: EFFECTS OF TANNIN AND CHOLATE

| Substrate protein | pH | Experimental treatments[a] | Tannin present[b] | Tannin/protein ratio (T/P) | Ninhydrin assay absorbance ratio[c] | |
|---|---|---|---|---|---|---|
| | | | | | at 30 min | at 60 min |
| BSA | 7.5 | NP; −C | TA | 0.1 | 2.69 | 2.28 |
| | | | | 1.0 | 0.12 | 0.19 |
| | | | Q | 0.1 | 2.53 | 2.29 |
| | | | | 2.0 | 0.46 | 0.38 |
| γ-Globulin | 8.5 | RD; −C | TA | 0.1 | 1.38 | 1.35 |
| | | | | 1.0 | 1.75 | 1.62 |
| | | | Q | 0.2 | 1.40 | 1.33 |
| | | | | 2.0 | 1.81 | 1.56 |
| Hemoglobin | 8.5 | RD; −C | TA | 0.1 | 1.40 | 1.31 |
| | | | | 1.0 | 1.60 | 1.32 |
| | | | Q | 0.2 | 1.00 | 1.00 |
| | | | | 2.0 | 1.46 | 1.09 |
| BSA | 8.5 | RD; −C | TA | 0.1 | 0.72 | 0.80 |
| | | | | 1.0 | 0.56 | 0.62 |
| | | | Q | 0.2 | 1.00 | 1.00 |
| | | | | 2.0 | 0.54 | 0.78 |
| BSA | 7.5 | RD; −C | TA | 0.1 | 0.56 | 0.69 |
| | | | | 1.0 | 0.60 | 0.58 |
| | | | Q | 0.2 | 0.82 | 0.91 |
| | | | | 2.0 | 0.50 | 0.53 |
| BSA | 6.2 | RD; +C[d] | TA | 0.1 | 0.93 | 0.93 |
| | | | | 1.0 | 0.47 | 0.54 |
| | | | Q | 0.1 | 0.93 | 0.91 |
| | | | | 1.0 | 0.57 | 0.62 |
| BSA | 6.2 | NP; −C[d] | | | 0.31 | 0.35 |
| BSA | 6.2 | P; −C[d] | TA | 0.1 | 0.64 | 0.76 |
| | | | | 1.0 | 0.14 | 0.09 |
| | | | Q | 0.1 | 0.66 | 0.74 |
| | | | | 1.0 | 0.20 | 0.16 |

[a]NP = not precipitated; RD = precipitated and redissolved; P = precipitated; +C = contains 8.0 mM cholate; −C = cholate absent.
[b]TA = tannic acid; Q = quebracho tannin.
[c]The ratio of the absorbances produced by the proteolysis products of experimental versus control treatments when sampled at the time indicated. Each ratio represents the mean of three replicate experiments.
[d]The control reaction contained 8.0 mM cholate.

It is suggested that enhanced and unaffected rates of tryptic digestion in the presence of tannins are dependent on the nature and native state of the protein substrate and the precise conditions under which it interacts with tannin. Recently, Martin and Martin (1984) have pointed out that the surfactants contained in insect digestive fluids, and their ability to hinder protein precipitation, must be taken into consideration in these studies. This point has been further illustrated by demonstrating the unfavorability of insect gut conditions for tannin–protein precipitation using a good *in vitro* simulation for a composite lepidopteran gut fluid based on four species (Martin et al., 1985).

Our observations suggest that Martin and Martin and others (Feeny, 1976; Rhoades and Cates, 1976; Hagerman and Butler, 1978) may be mistaken in correlating the absence of protein precipitation with noninterference of tannins with proteolysis. Nevertheless, we do not doubt that surfactants are important in protein digestion; this led us to investigatiate the modifying effects of the mammalian bile acid cholic acid on the systems already studied. We have found that a precipitate of BSA and tannic acid at pH 6.2 is redissolved by 8 mM cholate, a concentration which corresponds to that found in the human small intestine. (Sjoval, 1959). Once a precipitate with T/P = 1.0 is solubilized by this concentration of cholate, digestion proceeds at a much faster rate than in the absence of cholate, although there is still a marked inhibition in comparison to a control reaction (Table 1). At T/P = 0.1 tannic acid formed a precipitate with BSA which subsequently redissolved (van Buren and Robinson, 1969), but in this instance cholate did not greatly influence the rate of proteolysis. It is important to note that in the latter case proteolysis with and without cholate proceeded almost as fast as the control containing cholic acid, i.e., at an increased rate compared with the control without cholate. While seemingly unrecorded in the medical literature (Hofmann, 1968; Passmore and Robson, 1976), surfactants may have a role in denaturing proteins in normal mammalian digestion, and this may explain why no additional tannin-induced digestive enhancement is seen when cholate is used where tannins and proteins would otherwise complex.

The relative effects of surfactants from mammals and insects on proteolytic systems remain to be investigated. Mammalian gut surfactants are synthesized outside the gut, while those of insects appear to be generated in the lumen of the gut (Turunen and Kastari, 1979), potentially in the presence of tannins.

It is clearly of crucial importance to use a protein in its native undenatured state as the substrate in experiments of this type. Casein, used by Feeny (1969) in his original investigations of tannin–protein interactions, has a notably loose and randomly coiled tertiary structure (White et al., 1978), so that the absence of any observation of enhanced tryptic digestion by Feeny is not surprising. Ideally, studies in vitro attempting to investigate the role of tannins as digestion inhibitors should use plant proteins, particularly ribulosedisphosphate carbox-

ylase isolated as fraction-1 leaf protein. Feeny (1969) did attempt experiments with an acetone powder prepared from leaves, but the preparation of this material may well have caused the protein to become denatured as would the heat treatment method used in bulk preparation of leaf protein (Jones and Mangan, 1976). The most suitable commercial product (Sigma) has a carboxylase activity (0.01–0.03 units/mg) much less than that retained (1.48 units/mg) by freshly salted out material (Siegel and Lane, 1976). Thus, at present it does not appear that an acceptable preparation of fraction-1 leaf protein exists. Salting out would appear to be the method of choice for obtaining leaf protein for studies of this type, but so far we have isolated only small quantities and we have only been able to confirm, by turbidity measurements, the solubility of products of the tannin–product interaction at pH 8.5 (unpublished results).

There has been much recent debate on the effectiveness and mode of action of tannins as digestion inhibitors (Waterman, 1983). Zucker (1983) has suggested that hydrolyzable tannins are adapted to bind to particular proteins, and this may be illustrated by their ability to damage the gut lining of acridids (Bernays, 1978). Berenbaum (1983) shows a "toxic" physiological response to tannins in papilionids, and she also suggests some degree of counteradaptation to their digestion-inhibiting effects. Whatever their function, there is now considerable evidence that, while most herbivores avoid ingestion of large amounts of tannins, they are not the generally efficient digestion inhibitors they were once supposed to be.

The implications of these results are that ecologically relevant conditions could occur under which an herbivore can experience increased tryptic digestion from a diet containing a mixture of tannins and proteins when compared with a tannin-free diet with the same protein content. For example, half the species reported by Coley (1983) have foliar T/P ratios of less than 1.0 and could be considered as candidates for causing enhanced tryptic digestion in the gut of an insect herbivore! For some insects, reports attributing a phagostimulatory role to tannins and evidence of an ability of these animals to deal with and even thrive on (Bernays and Woodhead, 1982) diets rich in tannins are found in the literature (Bernays, 1981). Although mammalian digestion differs importantly in that the high gut pHs of some insects do not occur, it is of interest that Jones and Mangan (1977) have attempted to explain observations of increased nitrogen retention from tannin-containing diets in sheep on the basis of dissolution of tannin–protein complexes making protein available for tryptic digestion. The potential duplicity of the tannin–protein interaction clearly has enormous ecological significance and makes investigation in vivo of the enhancement effects of paramount importance.

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# NEW LEPIDOPTERAN SEX ATTRACTANTS FOUND BY SYSTEMATIC FIELD SCREENING OF BLENDS CONTAINING (*Z*)-11- AND (*E*)-11-TETRADECENAL

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**Abstract**—By screening (*Z*)-11-tetradecenal and (*E*)-11-tetradecenal alone, mixed with each other or mixed with corresponding alcohols or acetates, sex attractants were discovered or improved for nine Lepidoptera. Attractants were found for *Acleris sparsana*, *A. tripunctana*, *Aphelia viburnana* (all Tortricidae), *Cilix glaucata* (Drepanidae), and *Cosmia trapezina* (Noctuidae). The significance of Δ11-tetradecenals in tortricid sex attractants is discussed.

**Key Words**—(*Z*)-11-tetradecenal, (*E*)-11-tetradecenal, sex attractants, Tortricidae, Drepanidae, Noctuidae.

## INTRODUCTION

Many lepidopteran sex attractants consist of monounsaturated alkenyl acetates, alkenols, or alkenals with a straight chain of 12, 14, or 16 carbon atoms. Whereas the general significance of acetates and alcohols as attractive compounds has been known for about 20 years, the importance of aldehyde compounds was recognized much later. Alkenals were first found to be sex pheromone components in the early 1970's (Weatherston et al., 1971, 1974; Sanders et al., 1974), but their common occurrence in tortricid and noctuid sex attractants was established much later (Underhill et al., 1977; Weatherston et al., 1978; Ando et al., 1981).

So far, about 20 tortricid sex attractants are known, in which *Z*11–14 : Ald or *E*11–14 : Ald are major components. The attracted species mainly belong to the genera *Choristoneura*, *Acleris*, and *Croesia*. Most of these attractants were discovered in Japan (Ando et al., 1981) and Canada (Weatherston et al., 1971,



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1974, 1976). In Europe, however, the attractancy of Z11–14 : Ald and *E*11–14 : Ald to Lepidoptera has hardly been tested.

In an effort to get a better picture of tortricid sex attractants, we tested many synthetic compounds and mixtures in field trials in The Netherlands in 1982 and 1983 (Booij and Voerman, 1984a–d). In 1983, several series of blends with Z11–14 : Ald and *E*11–14 : Ald were tested.

## METHODS AND MATERIALS

*E*11–14 : OH and Z11–14 : OH were obtained by stirring the corresponding acetates in a solution of KOH in methanol (20 g/liter). After working up in the usual way, both compounds were purified by argentation chromatography with a glass column of 200 cm × 1.6 cm ID, packed with Lewatit SP 1080 ($Ag^+$), 170–200 mesh, with methanol as mobile phase (Houx et al., 1974). *E*11–14 : Ac and Z11–14 : Ac were purified through the same column. Ultimate purity of all four compounds was 99% or more as checked by GC and HPLC (Voerman, 1979; Houx and Voerman, 1976).

To prepare *E*11–14 : Ald, a solution of 100 mg of pure *E*11–14 : OH in 1 ml of $CH_2Cl_2$ was added to a slurry of 162 mg of pyridinium chlorochromate and 33 mg of sodium acetate in 1.5 ml $CH_2Cl_2$. After 4 hr of stirring, the mixture was diluted with 5 ml of ether and decanted. Again the mixture was shaken with some ether and decanted. The combined extracts were concentrated to 0.5 ml and chromatographed through a small column (8 mm ID) of 1.3 g Florisil 100–120 mesh, with 1 g of $Na_2SO_4$ at the bottom, using hexane as mobile phase. The eluate was checked by GC and the concentration of *E*11–14 : Ald was estimated with the alcohol as standard. The combined fractions were diluted with $CH_2Cl_2$ and the antioxidant 2,6-di-*tert*-butyl-4-methylphenol was added in an amount equal to the amount of aldehyde. Z11–14 : Ald was prepared in a similar way. The solutions were stored at −20°C. After a year no trimerization of these aldehydes could be detected by GC (Dunkelblum et al., 1984). All reactions and the liquid chromatography were monitored by GC, using a glass column of 2 m × 2 mm ID, packed with 2% SE-30 on Chromosorb W HP 80/100 (Corey and Suggs, 1975; Sonnet and Heath, 1980).

For each compound or blend of compounds to be tested, 1 mg was applied to red-rubber sleeve stoppers, 5 × 9 mm (Arthur H. Thomas Company, Philadelphia, Pennsylvania, catalog No. 1780-B10). Earlier tests had shown that aldehydes applied to the same septa remained attractive for at least two months, indicating no serious breakdown of the compounds. (However, see Steck et al., 1979.) The lures were fixed into the top of white delta traps with disposable sticky bottoms (10 × 17 cm). Tangle-Trap was used as sticky material (The Tanglefoot Co., Grand Rapids, Michigan). In the experimental areas, traps were fixed to tree branches about 2 m off the ground. Traps were checked weekly

and the moths identified, counted, and removed. Lures were renewed every four weeks.

The following lures were tested: Series A: Z11-14 : Ald, *E*11-14 : Ald, Z11-14 : Ald + *E*11-14 : Ald (1 : 1), Z11-14 : Ald + Z11-14 : Ac (1 : 1), *E*11-14 : Ald + *E*11-14 : Ac (1 : 1), Z11-14 : Ald + Z11-14 : OH (1 : 1), *E*11-14 : Ald + *E*11-14 : OH (1 : 1). Series B: Z11-14 : Ald + *E*11-14 : Ald (9 : 1 and 1 : 9), *E*11-14 : Ald + *E*11-14 : Ac (9 : 1 and 1 : 9).

Traps baited with these mixtures were tested in the following areas: (1) Loenen Estate, Valburg, a mixed deciduous forest on clay-loam. Series A: June–August (two replicates). (2) The Bruuk, Groesbeek, a mesotrophic fen area with alder and willow shrubs. Series A: June–September. Series B: August–September (two replicates). (3) Planken Wambuis, Ede, a dry forest area with oak, birch, and heath. Series A: July–September. Series B: August–September (two replicates). (4) The Schuilenburg experimental apple orchard, Lienden. Series A + B: September (one replicate).

## RESULTS AND DISCUSSION

In Table 1, the mean catches of male moths in traps baited with different lures are given. Six tortricid species, one gracillariid, one noctuid, and one drepanid species were caught. None of the species was trapped in blank control traps.

In particular, the attraction of the drepanid species, *Cilix glaucata*(Scopoli), was interesting, since no sex attractant was known to any species of that small family. We had occasionally caught this species before in traps baited with various mixtures containing *E*11-14 : Ac. Now the combination *E*11-14 : Ac + *E*11-14 : Ald was found to attract significant numbers of this species. The ratio 9 : 1, which was only tested from August onwards, seemed to be more attractive than the ratio 1 : 1.

The gracillariid moth *Gracillaria syringella* (F.) was maximally attracted to *E*11-14 : Ald alone. When it was mixed with *E*11-14 : OH, captures were reduced. With *E*11-14 : Ald + Z11-14 : Ald (1 : 1), no males were caught at all, suggesting an inhibitory effect of Z11-14 : Ald, although Ando et al. (1981) reported the attraction of some males to *E*11-14 : Ald + Z11-14: Ald (9 : 1) and none to *E*11-14 : Ald alone.

The noctuid moth, *Cosmia trapezina* (L.), was attracted in small numbers to traps baited with Z11-14 : Ald alone (two traps, 10 males) and with Z11-14 : Ald + *E*11-14 : Ald (1 : 1) (two traps, seven males). Since monounsaturated E compounds are known to be attractive only to a few noctuid species (Steck et al., 1982), Z11-14 : Ald is likely to be the main attractive compound. By adding structurally related compounds like Z9-14 : Ald or Z11-16 : Ald, a more attractive blend may be found.

TABLE 1. MEAN NUMBER OF MALE MOTHS CAUGHT IN TRAPS BAITED WITH DIFFERENT LURES AT LOENEN (1), GROESBEEK (2), EDE (3), AND LIENDEN (4), THE NETHERLANDS, JUNE–SEPTEMBER, 1983

| Species | Lure | Mean number per trap | Area |
|---|---|---|---|
| Tortricidae | | | |
| *Acleris sparsana* | *E*11–14:Ald + Z11–14:Ald (9:1) | 6.6[a] | 2, 3, 4 |
| *Acleris tripunctana* | *E*11–14:Ald + Z11–14:Ald (1:1) | 126.0[b] | 3 |
| *Eulia ministrana* | Z11–14:Ald + Z11–14:Ac (1:1) | 59.5[b] | 3 |
| *Aphelia viburnana* | Z11–14:Ald | 5.5[b] | 3 |
| *Choristoneura hebenstreitella* | Z11–14:Ald + Z11–14:OH (1:1) | 35.8[c] | 2, 3 |
| *Aleimma loeflingiana* | *E*11–14:Ald + *E*11–14:Ac (1:1) | 4.8[c] | 1, 2 |
| Gracillariidae | | | |
| *Gracillaria syringella* | *E*11–14:Ald | 164.5[b] | 1 |
| | *E*11–14:Ald + *E*11–14:OH (1:1) | 114.0[b] | 1 |
| Drepanidae | | | |
| *Cilix glaucata* | *E*11–14:Ac + *E*11–14:Ald (9:1) | 16.5[b] | 2 |
| | *E*11–14:Ac + *E*11–14:Ald (1:1) | 6.0[b] | 2 |
| Noctuidae | | | |
| *Cosmia trapezina* | Z11–14:Ald (+ *E*11–14:Ald) | 4.3[c] | 2, 3 |

[a] Mean of 5 traps.
[b] Mean of 2 traps.
[c] Mean of 4 traps.

The mixture Z11–14 : Ald + Z11–14 : OH (1 : 1) strongly attracted males of *Choristoneura hebenstreitella* (Müller). In 1982, we caught 19 males in two traps baited with Z11–14 : OH alone. The large numbers caught in 1983 with Z11–14 : Ald + Z11–14 : OH suggest that Z11–14 : Ald improves attractancy, but further comparative tests are necessary. Several *Choristoneura* species are attracted by aldehyde compounds (Weatherson et al., 1971, 1978; Sanders and Weatherston, 1976; Daterman et al., 1977).

*Aleimma loeflingiana* (L.) was attracted in small numbers to *E*11–14 : Ac + *E*11–14 : Ald (1 : 1). In other trials this species was strongly attracted to *E*11–14 : Ac + Z11–14 : Ac (8 : 2) but hardly to *E*11–14 : Ac alone. The results of both trials suggest that *E*11–14 : Ac is the main attractant and that both *E*11–14 : Ald and Z11–14 : Ac may be important coattractants.

To traps baited with Z11–14 : Ald alone, 11 males of *Aphelia viburnana* (D. & S.) were attracted. Since this species is rather uncommon in The Netherlands, and males were caught in two independent traps on two successive dates, we regarded this capture as significant, despite the small total number of males caught.

The attraction of *Eulia ministrana* (L.) to Z11–14 : Ald + Z11–14 : Ac (1 : 1) is quite interesting, because it is the first tortricid species of the tribe Cnephasiini for which an aldehyde appears to be attractive. In 1982, the species was attracted in about equal numbers to Z11–14 : Ac + Z9–14 : Ac (9 : 1), but not at all to Z11–14 : Ac alone. The combined results indicate that a mixture of Z11–14 : Ac + Z11–14 : Ald + Z9–14 : Ac may be optimally attractive.

Since several *Acleris* species are known to be attracted to Δ11-tetradecenals, the attraction of two other *Acleris* species was not surprising. For all species, *E*11–14 : Ald seemed to be the main attractive compound. To *E*11–14 : Ald alone, *A. emargana* (F.) (Weatherston et al., 1974) and *A. scabrana* (D. & S.) (Tagestad, 1975) are attracted; the combination with Z11–14 : Ald attracted three species: *A. perfundana* (Kuznetsou) (Ando et al., 1981), *A. sparsana* (D. & S.), and *A. tripunctana* (Hübner) (this paper). *Acleris filipjevi* (Obraztsov) and *A. emitescens* (Meyrick) are weakly attracted to *E*11–14 : Ald + *E*11–14 Ac (Ando et al., 1981). Finally a diunsaturated aldehyde, *E*11,13–14. Ald, was identified as a sex pheromone of *A. minuta* (Robinson) (Schwartz et al., 1983).

The utilization frequency of aldehydes as sex attractants within the family Tortricidae is striking in that they only attract species of the subfamily Tortricinae. In the Olethreutinae, where most species are attracted by C-12 compounds, only alcohols and acetates have been reported as attractive compounds so far (Roelofs and Brown, 1982; Inscoe, 1982).

Within the Tortricinae, aldehydes are most frequently found to attract species of the tribes Tortricini and Archipini. In the Tortricini, aldehydes are the main component in the majority of the known sex attractants (14 species of 22). Aldehyde-using species mainly belong to the genera *Acleris* and *Croesia*. In the large tribe Archipini, sex attractants are known for about 100 species, but only 11 of these are attracted by aldehydes, most of them belonging to the genus *Choristoneura* (seven species). In another large genus of this tribe, the genus *Archips*, only *A. griseus* Robinson is known to use aldehydes (Grant and Slessor, 1983), whereas about 20 *Archips* species are only attracted by acetates. Thus there is an almost complete divergence between these genera.

The recognition that aldehydes are an important group of attractant compounds in Tortricinae may be helpful in finding more sex attractants for this group by field screening tests.

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# 9,11-DODECADIENYL ALCOHOLS, ACETATES, OR ALDEHYDES AS SYNTHETIC CHEMICAL SEX ATTRACTANTS FOR FOUR LEPIDOPTERA: *Cosmopterix gemmiferella* (Clemens), *Dichrorampha simulana* (Clemens), *Tortricidia testacea* (Packard), and an *Ancylis* Sp.

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**Abstract**—Four lepidoptera were lured to field traps containing various combinations of 9,11-dodecadienes with alcohol, acetate, or aldehyde functional groups. All species required two chemical components for best attraction. *Cosmopterix gemmiferella* was most responsive to a combination of (9*Z*)-9,11-dodecadienyl acetate and (9*E*)-9,11-dodecadienyl acetate in 2:1 ratio; *Dichrorampha simulana* to (9*E*)-9,11-dodecadienyl acetate and (9*E*)-9,11-dodecadien-1-ol in 10:1 ratio; *Tortricidia testacea* to (9*Z*)-9,11-dodecadienal and (9*E*)-9,11-dodecadienal in 10:1 ratio; and *Ancylis* sp. to (9*Z*)-9,11-dodecadienyl acetate and (9*Z*)-9,11-dodecadien-1-ol in 10:1 ratio.

**Key Words**—Sex attractant, Cosmopterigidae, Tortricidae, *Cosmopterix gemmiferella, Dichrorampha simulana, Tortricidia testacea, Ancylis* sp., (9*Z*)-9,11-dodecadienyl acetate, (9*E*)-9,11-dodecadienyl acetate, (9*Z*)-9,11-dodecadien-1-ol, (9*E*)-9,11-dodecadien-1-ol, (9*Z*)-9,11-dodecadienal, (9*E*)-9,11-dodecadienal.

## INTRODUCTION

During 1979 to 1984 we carried out a field survey of the attractancy of both geometrical isomers of 9,11-dodecadienes with acetate, alcohol, and aldehyde functional groups for male moth species occurring in western Canada. As a



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result of that survey, sex lures were obtained for four moths: *Cosmopterix gemmiferella* (Clemens) (Cosmopteriginae), *Dichrorampha simulana* (Clemens) (Tortricidae), *Tortricidia testacea* Pack (Limacodidae) and an *Ancylis* sp. (Tortricidae).

The genus *Cosmopterix* contains more than 140 species worldwide, with 30 species occurring in North America north of Mexico, but no species have been reported from northwestern North America. The larvae mine the leaves of plants in the families Gramineae, Leguminosae, Cyperaceae, and Compositae (Hodges, 1978). In Japan Ando et al. (1975, 1977) have reported that (*Z*)-7-dodecenyl acetate (*Z*7–12 : Ac) attracts *C. fulminella,* (*Z*)-10-tridecenyl acetate (Z10–13 : Ac) attracts *C. victor,* and (*Z*)-9-dodecenyl acetate (*Z*9–12 : Ac) attracted four unidentified species of *Cosmopterix.*

Hodges et al. (1983) list 14 species in the genus *Dichrorampha,* but Miller (1983) has grouped eight of these into three species, and he considers *D. capatana* (Busck), *D. britana* (Busck) and *D. kana* (Busck) as synonyms of *D. simulana* (Clemens). Aster species have been reported as host plants for *D. simulana* (McDunnough, 1946). Two monoene lures have been reported as sex attractants for *Dichrorampha:* (*E*)-8-dodecenyl acetate (*E*8–12 : Ac) + (*E*)-8-dodecen-1-ol (*E*8–12 : OH) for an unidentified species (Roelofs and Comeau, 1971) and (*E*)-9-dodecenyl acetate (*E*9–12 : Ac) + (*Z*)-9-dodecenyl acetate (*Z*9–12 : Ac) for *D. plumbana* (Scapoli) (Booij and Voerman, 1984).

Sex attractant chemical lures have been reported from the Netherlands for three species of *Ancylis: A. epicella* (Den & Schiff) was attracted to a combination of (*Z*)-8-dodecenyl acetate (Z8–12 : Ac) + (*Z*)-9-dodecenyl acetate (*Z*9–12 : Ac) and *A. geminana* (Donovan) and *A. uncella* (Den & Schiff) were both attracted by (*Z*)-9-dodecen-1-ol (*Z*9–12 : OH) (Booij and Voerman, 1984). We are not aware of sex attractant reports for any of the North American *Ancylis* sp. listed by Hodges et al. (1983).

The larvae of *T. testacea* feed on oak, birch, and cherry trees (Forbes, 1923). However, our computer search of the literature (1969–1984) gave no citation for this genus.

We report here synthetic chemical sex attractants for *C. gemmiferella, D. simulana, Tortricidia testacea,* and an *Ancylis* sp.

## METHODS AND MATERIALS

The chemicals used in this study were synthesized in this Laboratory. A mixture of (9*E*)-9,11-dodecadienyl acetate (9*E*,11–12 : Ac) and (9*Z*)-9,11-dodecadienyl acetate (9*Z*,11–12 : Ac) was obtained by Wittig condensation reaction (Nesbitt et al., 1973b). After initial purification, the acetate function was removed by hydrolysis (methanolic KOH) and the (9*E*)-9,11-dodecadien-1-ol (9*E*,11–12 : OH) was separated from 9Z,11–12 : OH by elution from a silver-loaded

resin column (2 × 69 cm) with methanol (Houx et al., 1974). This column gave baseline separation of the two geometrical isomers of the dien-1-ols. A Hewlett-Packard gas chromatograph with flame-ionization detector and equipped with either a DB-1 glass column (30 m × 0.3 mm ID; J and W Scientific Inc., Rancho Cordova, California) or a liquid-crystal-phase glass column (36 m × 0.25 mm ID) (Heath et al., 1979, 1981) was used to monitor the fractions from the silver resin column. Acetylation of a portion of each dienyl alcohol with 10 parts of $Ac_2O$ and 1 part of anhydrous NaOAc (90°C, 30 min) gave quantitative yields of the corresponding acetates. Another portion of each dienyl alcohol was oxidized to the corresponding aldehyde with pyridinium chlorochromate (Corey and Suggs, 1975). All compounds gave expected IR and PMR spectra.

In both GC columns the *Z* isomer of the 9,11-dodecadienes with aldehyde, alcohol, or acetate functions eluted first. The DB-1 column programmed from 90° to 250°C at 4°/min gave peak retention time differences, but incomplete separation. The liquid-crystal column operated isothermally at 160°C gave baseline separation of the *Z* and *E* isomers of the dienes with all three functional groups.

Field trapping was carried out in two locations. At one location near Saskatoon, Canada (52.3°N, 106.5°W) the traps, spaced 15 m apart, were hung on a fence-line near the top of the canopy of the vegetation. The fence which separated a wheat field from an experimental farm was immediately surrounded by a mixture of unidentified native and domestic grasses. The other location was 100 km northeast of Saskatoon in an undisturbed forest composed of spruce, pine, aspen, and birch trees. The trees were interspersed with small meadows and an assortment of herbaceous shrubs. The traps were hung on tree branches 1–2 m above the ground and were spaced 15–20 m apart.

Pherocon ICP traps (Zoecon Corp., Palo Alto, California), hung in a randomized block design, were baited with lures prepared by adding appropriate concentrations of chemical in hexane solution to pink rubber septa (Arthur H. Thomas #8753-D22). Each lure was routinely protected by adding two drops of a 10% solution of the antioxidant butylated hydroxytoluene (BHT) in acetone to the septa. The traps were examined twice weekly, the numbers of moths captured were recorded, and then the moths were removed from the sticky trap liners.

The replicated field data were transformed $\sqrt{X + 0.5}$, where $X$ is the number of male moths captured per trap, and then analyzed by analysis of variance; significantly different means were separated by Duncan's multiple-range test.

## RESULTS AND DISCUSSION

In the initial survey, each trap was baited with 100 μg of a single 9,11-dodecadiene. Traps containing 9Z,11-12:Ac captured 14 *C. gemmiferella*,

9*E*,11–12 : Ac captured 17 *D. simulana*, (9*Z*)-9,11-dodecadienal (9*Z*,11–12 : Ald) captured 29 *T. testacea*, and 9*Z*,11–12 : Ac captured 51 *Ancylis* sp. Subsequent replicated field test data for the four moths are presented in Tables 1–4.

Table 1 shows that 9*Z*,11–12 : Ac + 9*E*,11–12 : Ac in 2 : 1 ratio is an effective attractant for *C. gemmiferella*. Fewer moths were caught with lower or higher ratios of 9*E*,11–12 : Ac. The 9*E*,11–12 : Ac when tested alone in the original survey or in Table 1 captured no moths. The 9*Z*,11–12 : Ac alone, which captured a number of *C. gemmiferella* in the initial survey, was not an effective lure when placed in competition with the two-component lure in 2 : 1 ratio (Table 1).

*D. simulana*, the *Ancylis* sp., and *T. testacea* (Tables 2–4) are narrowly sympatric. All three species are attracted to two-component lures composed of 9,11-dodecadienes and both *D. simulana* and the *Ancylis* sp. use the same functional groups. But *D. simulana* uses the *E* isomer, while the *Ancylis* sp. uses the *Z* isomer. In additional field tests using *E*9–12 : Ac, *Z*9–12 : Ac, Δ11–12 : Ac and *Z*9–12 : OH alone or in various combinations with the dienes had no effect on capture of *D. simulana* or the *Ancylis* sp. The best lure tested for attraction of *T. testacea* was a combination of (9*E*)-9,11-dodecadienal (9*E*,11–12 : Ald) and 9*Z*,11–12 : Ald. In a separate test, (9*Z*)-9,11-dodecadien-1-ol (9*Z*,11–12 : OH), 9*Z*,11–12 : Ac, and 9*E*,11–12 : Ac were shown to inhibit attraction of *T. testacea*.

TABLE 1. CAPTURE OF *Cosmopterix gemmiferella* BY TRAPS BAITED WITH 9,11-DODECADIENES

| Lure composition ($\mu$g) | Total males captured[a] |
|---|---|
| 9*Z*,11–12:Ac (10) | 1 c |
| 9*Z*,11–12:Ac (100) | 1 c |
| 9*Z*,11–12:Ac (1000) | 2 c |
| 9*Z*,11–12:Ac (100) + 9*E*,11–12:Ac (1) | 4 c |
| 9*Z*,11–12:Ac (100) + 9*E*,11–12:Ac (10) | 50 b |
| 9*Z*,11–12:Ac (100) + 9*E*,11–12:Ac (50) | 124 a |
| 9*Z*,11–12:Ac (100) + 9*E*,11–12:Ac (100) | 2 c |
| 9*Z*,11–12:Ac (100) + 9*Z*,11–12:OH (50) | 0 c |
| 9*Z*,11–12:Ac (100) + 9*Z*,11–12:Ald (50) | 4 c |
| 9*Z*,11–12:Ac (100) + 9*E*,11–12:OH (50) | 2 c |
| 9*Z*,11–12:Ac (100) + 9*E*,11–12:Ald (50) | 2 c |
| 9*Z*,11–12:OH (100) | 1 c |
| 9*Z*,11–12:Ald (100) | 6 c |
| 9*E*,11–12:Ac (100) | 0 c |
| 9*E*,11–12:OH (100) | 0 c |
| 9*E*,11–12:Ald (100) | 0 c |

[a] 3X replicated, 21 July to 8 August, 1980. Numbers followed by the same letter are not different ($P = 0.05$).

TABLE 2. CAPTURE OF *Dichrorampha simulana* BY TRAPS BAITED WITH 9,11-DODECADIENES

| Lure composition ($\mu$g) | Total males captured[a] |
|---|---|
| 9*E*,11-12:Ac (100) | 1 c |
| 9*E*,11-12:Ac (100) + 9*Z*,11-12:Ac (10) | 1 c |
| 9*E*,11-12:Ac (100) + 9*E*,11-12:OH (10) | 46 a |
| 9*E*,11-12:Ac (100) + 9*E*,11-12:Ald (10) | 15 b |
| 9*Z*,11-12:Ac (100) | 0 c |

[a]3X replicated, 12 July to 2 August, 1983. Numbers followed by the same letter are not different ($P = 0.05$).

TABLE 3. CAPTURE OF *Ancylis* SP. BY TRAPS BAITED WITH 9,11-DODECADIENES

| Lure composition ($\mu$g) | Total males captured[a] |
|---|---|
| 9*Z*,11-12:Ac (100) | 10 b |
| 9*Z*,11-12:Ac (100) + 9*Z*,11-12:OH (10) | 51 a |
| 9*Z*,11-12:Ac (100) + 9*Z*,11-12:Ald (10) | 10 b |
| 9*Z*,11-12:Ac (100) + 9*E*,11-12:Ac (10) | 4 b |

[a]3X replicated, 16 June to 14 July, 1983. Numbers followed by the same letter are not different ($P = 0.05$).

TABLE 4. CAPTURE OF *Tortricidia testacea* BY TRAPS BAITED WITH 9,11-DODECADIENES

| Lure composition ($\mu$g) | Total male captured,[a] test A |
|---|---|
| 9*Z*,11-12:Ald (100) | 29 b |
| *Z*9-12:Ald (100) | 0 b |
| 11-12:Ald (100) | 0 b |
| 9*Z*,11-12:Ald (100) + 9*E*,11-12:Ald (10) | 250 a |
| 9*Z*,11-12:Ald (100) + 9*Z*,11-12:OH (10) | 2 b |
| 9*Z*,11-12:Ald (100) + 9*Z*,11-12:Ac (10) | 0 b |
| 9*Z*,11-12:Ald (100) + *Z*9-12:Ald (10) | 1 b |

[a]3X replicated, 22 June to 13 July, 1984. Numbers followed by the same letter are not different ($P = 0.05$).

Consequently geometrical isomerism and functional groups may play a role in separation of these three species.

We are aware of five other insects that use 9,11-dodecadienes as sex attractants. Three have been shown to be pheromones: *Diparopis castanea* (Nesbitt et al., 1973a, 1975), *Sparganothis directana* (Bjostad et al., 1980), and *Rhyacionia frustrana* (Hill et al., 1981). In Europe *Dichrorampha gueneeana* Obraztsov and *D. acuminatana* (Lienig and Zeller) are known to be attracted to 9*Z*,11-12:Ac (C.J.H. Booij, personal communication).

The sex attractants reported here may be of value for detection or study of: *C. gemmeriferella*, 9*Z*,11-12:Ac + 9*E*,11-12:Ac in 2:1 ratio; *D. simulana*, 9*E*,11-12:Ac + 9*E*,11-12:OH in 10:1 ratio; *T. testacea*, 9*Z*,11-12:Ald + 9*E*,11-12:Ald in 10:1 ratio; and for some species of *Ancylis*, 9*Z*,11-12:Ac + 9*Z*,11-12:OH in 10:1 ratio.

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# SYNERGISM BETWEEN MYRISTICIN AND XANTHOTOXIN, A NATURALLY COOCCURRING PLANT TOXICANT

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**Abstract**—Myristicin, a methylenedioxyphenyl (MDP)-containing phenylpropene constituent of the leaves of many plants in the family Umbelliferae, is a highly effective synergist of the cooccurring furanocoumarin xanthotoxin. As little as 0.10% in an artificial diet can increase the toxicity of xanthotoxin to *Heliothis zea* (Lepidotera: Noctuidae) fivefold. In addition to increasing the proportion of caterpillars dying at a given xanthotoxin concentration, myristicin also increases the rate at which they die and increases the time to molt of surviving larvae. That there was no increase in the deterrency of xanthotoxin in the presence of myristicin suggests that the mechanism of synergism is not behaviorial but rather is biochemical, via MDP competitive inhibition of microsomal mixed function oxidases.

**Key Words**—Methylenedioxyphenyl compounds, myristicin, piperonyl butoxide, synergism, Umbelliferae, xanthotoxin, furanocoumarins, *Heliothis zea*, Lepidoptera, Noctuidae, insect–plant interaction.

## INTRODUCTION

Methylenedioxphenyl (MDP) compounds are naturally occurring plant secondary substances with the distinctive ability to synergize synthetic organic insecticides (Fuhremann and Lichtenstein, 1979; Casida, 1970; Lichtenstein et al., 1974; Wilkinson, 1976). Their mode of action is to inhibit the activity of mixed-function oxidases (MFO), the enzymes responsible for the metabolism of many lipophilic xenobiotics in both vertebrates and invertebrates (Casida, 1970; Wilkinson, 1973; Chang et al., 1981). In many insects, the MFO system is the principal means by which insects detoxify naturally occurring plant toxicants (Brattsten et al., 1977; Brattsten, 1979a, b).



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MDP compounds are widespread among plants (Newman, 1962) and cooccur with many classes of secondary compounds known to have insecticidal properties. The possibility exists, then, that MDPs act as natural synergists, interfering with MFO function after ingestion by insects to render them more susceptible to the toxic activity of other secondary substances. This mechanism was postulated to operate in *Chrysanthemum cinerariaefolium* (Compositae), in which the MDP synergist sesamin cooccurs with insecticidal pyrethrins in the flowerheads (Krieger et al., 1971). Production of such synergists may allow plants to reduce the quantity of secondary substances produced without reducing overall toxicity (Janzen, 1973).

If MDPs act as natural synergists of plant toxicants, the following criteria pertain: (1) synergists themselves will display little or no toxicity (Wilkinson, 1973); (2) the insecticidal properties of naturally occurring secondary substances will be enhanced in the presence of MDPs at natural concentrations (provided that the secondary substances are metabolized by MFO enzymes); (3) equivalent toxicity can be achieved with high levels of toxin and low levels of synergist or low levels of toxin and high levels of synergist.

The Umbelliferae is an ideal group in which to examine possible interactions among natural toxicants and insecticide synergists. Plants within the family produce a great diversity of MDPs (Newman, 1962) with known synergistic properties (Lichtenstein et al., 1974; Fuhremann and Lichtenstein, 1979). Many of these same plants also produce secondary substances with known insecticidal properties. Among these are furanocoumarins, benz-2-pyrone compounds produced principally by species in the subfamily Apioideae (Heywood, 1971). Furanocoumarins owe their toxicity to insects at least in part to UV-mediated cross-linkage of opposing DNA strands and subsequent interference with transcription and template formation (Berenbaum, 1978; Wat et al., 1981). However, there are toxic effects of furanocoumarin ingestion that are independent of UV light as well (Berenbaum, 1978); the mechanism of dark-toxicity is as yet unknown (Murray et al., 1982).

In this study, we examined the effects of independent and simultaneous ingestion of a purported MDP synergist and a known insect toxicant cooccurring widely within the Umbelliferae. Myristicin was selected as a representative MDP. It is widespread among plants in the family, occurring in over 20 species (Harborne et al., 1969), and is the principal MDP in many of the species in which it does occur (Kubeczka and Stahl, 1975; Bohannon and Kleiman, 1977; Ashraf et al., 1979, Stahl and Kubeczka, 1979). Xanthotoxin, a representative furanocoumarin, has been identified as an insecticide (Berenbaum, 1978; Yajima et al., 1977; Wat et al. 1981) and is widespread among species in the tribes Apieae and Peucedaneae (Murray et al., 1982), principal producers of MDPs. In addition, xanthotoxin is metabolized by midgut oxidation reactions in at least two species of Lepidoptera (Ivie et al., 1983); this pattern of detoxification is

consistent with MFO metabolism, thus making xanthotoxin a likely candidate for synergistic inhibition by MDPs.

## METHODS AND MATERIALS

*Chemicals.* Myristicin was purchased from Saber Labs (Morton Grove, Illinois); xanthotoxin was purchased from Sigma (St. Louis, Missouri). Piperonyl butoxide (PB) was obtained from K & K (Plainview, New York).

*Bioassay.* The insect selected for bioassay was *Heliothis zea*, the corn earworm (Lepidoptera: Noctuidae), a broadly polyphagous species with several umbellifers included among its over 100 recorded host plants (Tietz, 1972; references in Kogan et al., 1978). Chemicals were administered in a series of semisynthetic diets containing wheat germ as the only undefined ingredient (Appendix 1). The use of a meridic diet reduces the number of unidentified plant components with potential for synergistic interactions with the added allelochemicals. Four concentrations of myristicin (0, 0.01, 0.03, and 0.10%) were administered in combination with a range of dosages of xanthotoxin (0, 0.10, 0.25, 0.375, 0.50, 1.5, and 2.0% wet weight) until 90% or greater mortality was achieved. These levels parallel those reported to occur in a number of umbellifers (Berenbaum, 1981; Wulf et al., 1978; Yates et al., 1983; Franz and Gläsl, 1976). In a second series of experiments, piperonyl butoxide was incorporated into artificial diet containing 0.5% xanthotoxin at concentrations identical to those used for myristicin.

Neonate caterpillars from a laboratory culture (30 per treatment) were reared individually on diet cubes approximately 1.3 × 1.3 cm in size through first instar in 1-oz plastic cups (Bio-Serv, Frenchtown, New Jersey) under standard conditions (16/8 photoperiod, 25°C). The Sylvania F40 Cool-White 40-W fluorescent bulbs used for illumination produce no appreciable quantities of light in wavelengths less than 400 nm (as measured by a Blak-Ray longwave UV meter), so only the "dark-toxicity" of xanthotoxin, i.e., toxicity independent of UV-activated cross-linkage of DNA (Berenbaum, 1978; Murray et al., 1982), was measured under these conditions. Performance on the artificial diet, as measured by survivorship, time of death, and number of days to second instar, was checked daily and the different diets compared among treatments. Larvae off the diet when checked were returned to the cube surface; the number off the diets after 24 h was compared as a measure of deterrency of the different diets. $LC_{50}$ values were calculated for xanthotoxin at all four myristicin concentrations by standard techniques (Van der Waerden, 1969); differences in time to death and duration of second instar in surviving larvae were evaluated by two-way analysis of variance (ANOVA) with myristicin and xanthotoxin concentrations as main effects; and the proportions of larvae off the various diets were compared by Friedman's analysis of variance by ranks (Hays, 1973).

## RESULTS

At no dose level did myristicin alone produce mortality in neonate *H. zea* significantly greater than mortality on artificial diet to which no allelochemicals had been added (Table 1). Unsynergized xanthotoxin has an $LC_{50}$ of 0.96 g/100 g diet. In the presence of myristicin, xanthotoxin toxicity to first instar *H. zea* increased dramatically; synergistic ratios ($LC_{50}$ unsynergized xanthotoxin/$LC_{50}$ synergized xanthotoxin) for 0.01%, 0.03%, and 0.10% myristicin were 1.85, 2.54, and 4.97, respectively (Table 1). Equivalent mortality (approximately 90%), for example, is achieved with 2.0% unsynergized xanthotoxin or with 0.25% xanthotoxin + 0.10% myristicin.

A two-way ANOVA of the effects of xanthotoxin and myristicin at various concentrations on the rate at which first instar *H. zea* die (Table 2) yielded no significant main effects, but the interaction term was significant ($P = 0.03$). In other words, neither xanthotoxin nor myristicin dosage independently affected the rate at which the larvae died; however, the joint effect of increasing both xanthotoxin and myristicin was a decrease in the time to death. For larvae surviving to the second instar, two-way ANOVA revealed a significant effect of xanthotoxin on the duration of the first instar (Table 3); higher concentrations prolong the development time ($P = 0.0001$). Independently, myristicin has no effect on the duration of the first instar. The interaction term was highly significant ($P = 0.0077$); that is, the effect of xanthotoxin on prolonging development is exacerbated in the presence of myristicin.

As for feeding deterrency of the allelochemicals, a comparison of diets containing xanthotoxin with those lacking xanthotoxin showed that a significantly higher proportion of larvae were off diets containing xanthotoxin after 24 h (Table 4, Mann-Whitney test). However, for those diets containing xantho-

TABLE 1. MORTALITY (%) OF FIRST INSTAR *Heliothis zea* ON ARTIFICIAL DIETS

| Xanthotoxin (% wet wt) | Myristicin (% wet wt) | | | |
|---|---|---|---|---|
| | 0 | 0.01 | 0.03 | 0.10 |
| 0.000 | 1.1 | 0.0 | 0.0 | 0.0 |
| 0.100 | 0.0 | 6.7 | 0.0 | 6.7 |
| 0.250 | 20.0 | 13.3 | 43.3 | 86.7 |
| 0.375 | 23.3 | 40.0 | 38.5 | 93.3 |
| 0.500 | 16.7 | 30.0 | 60.0 | 86.7 |
| 1.500 | 50.0 | 100.0 | 96.7 | |
| 2.000 | 86.7 | | | |
| $LC_{50}$ | 0.96 | 0.57 | 0.38 | 0.19 |
| ± 95% confidence | (0.79–1.17) | (0.48–0.67) | (0.32–0.46) | (0.17–0.20) |
| Synergistic ratio | 1.00 | 1.85 | 2.54 | 4.97 |

TABLE 2. TIME TO DEATH (DAYS ± SD) OF FIRST INSTAR *Heliothis zea* ON DIETS WITH VARYING CONCENTRATIONS OF ALLELOCHEMICALS

| Xanthotoxin (% wet wt) | Myristicin (% wet wt) | | | |
|---|---|---|---|---|
| | 0 | 0.01 | 0.03 | 0.10 |
| 0.000 | 9.00 ± 3.70 | [a] | [a] | [a] |
| 0.100 | [a] | 5.50 ± 2.12 | [a] | 5.00 ± 0 |
| 0.250 | 3.83 ± 2.80 | 2.25 ± 0.50 | 3.38 ± 1.56 | 2.85 ± 0.54 |
| 0.375 | 2.71 ± 0.90 | 3.42 ± 1.62 | 2.50 ± 0.53 | 2.93 ± 0.59 |
| 0.500 | 5.40 ± 1.34 | 4.11 ± 1.90 | 3.00 ± 1.03 | 2.61 ± 0.90 |
| 1.500 | 4.86 ± 2.18 | 2.97 ± 0.67 | | |
| 2.000 | 3.50 ± 1.03 | | | |

[a] All caterpillars survived to second instar.

TABLE 3. DURATION (DAYS ± SD) OF FIRST INSTAR IN *Heliothis zea* SURVIVING TO MOLT ON ARTIFICIAL DIETS WITH VARYING CONCENTRATIONS OF ALLELOCHEMICALS

| Xanthotoxin (% wet wt) | Myristicin (% wet wt) | | | |
|---|---|---|---|---|
| | 0 | 0.01 | 0.03 | 0.10 |
| 0.000 | 3.07 ± 0.36 | 3.50 ± 0.51 | 3.13 ± 0.34 | 3.30 ± 0.47 |
| 0.100 | 4.53 ± 0.57 | 4.71 ± 0.53 | 4.43 ± 0.73 | 4.64 ± 0.68 |
| 0.250 | 4.21 ± 0.98 | 3.85 ± 1.08 | 4.82 ± 1.47 | 5.0 ± 0.0 |
| 0.375 | 4.48 ± 1.44 | 4.67 ± 1.28 | 4.75 ± 1.39 | 6.0 ± 0.0 |
| 0.500 | 5.96 ± 1.19 | 6.09 ± 0.94 | 6.50 ± 1.31 | 6.50 ± 1.00 |
| 1.500 | 8.82 ± 1.38 | [a] | | |
| 2.000 | 7.75 ± 0.96 | | | |

[a] No caterpillars survived to second instar.

TABLE 4. PROPORTION OF *Heliothis zea* OFF DIET CUBES WITH ADDED ALLELOCHEMICALS AFTER 24 HOURS (% OF TOTAL; $N$ = 30/treatment)

| Xanthotoxin (% wet wt) | Myristicin (% wet wt) | | | |
|---|---|---|---|---|
| | 0 | 0.01 | 0.03 | 0.10 |
| 0.000 | 7 | 7 | 10 | 13 |
| 0.100 | 57 | 73 | 23 | 27 |
| 0.250 | 40 | 37 | 40 | 83 |
| 0.375 | 23 | 40 | 53 | 77 |
| 0.500 | 63 | 66 | 53 | 57 |
| 1.500 | 66 | 77 | | |
| 2.000 | 63 | | | |

TABLE 5. MORTALITY (%) OF NEONATE *Heliothis zea* ON ARTIFICIAL DIET CONTAINING 0.5% XANTHOTOXIN AND SYNERGISTS

| Concentration of Synergist (% fresh wt) | Myristicin | Piperonyl butoxide |
|---|---|---|
| 0 | 16.7 | 16.7 |
| 0.01 | 30.0 | 63.3 |
| 0.03 | 60.0 | 60.0 |
| 0.10 | 86.7 | 53.3 |
| Slope of regression | 655.9 | 163.5 |
| $r$ = | 0.945 | 0.003 |

toxin, there was no significant effect of xanthotoxin concentration on the proportion of larvae off the diet, nor was there any effect of myristicin alone on the proportion of larvae off the diet (Friedman's two-way ANOVA by ranks) (Hays, 1973).

At high doses, myristicin is a more efficient synergist than piperonyl butoxide (Table 5), effecting almost 20% greater morality at a concentration of 0.10%. The synergistic relationship between myristicin and xanthotoxin appears to be linearly dose-dependent in the ranges tested, while PB is not ($r$ = 0.945 for myristicin and xanthotoxin vs. $r$ = 0.003 for PB and xanthotoxin).

## DISCUSSION

According to standard criteria, myristicin is a synergist of xanthotoxin in that it has no toxicity to the bioassay species *Heliothis zea* but enhances xanthotoxin toxicity almost fivefold. In previous studies (Lichtenstein and Casida, 1963; Lichstenstein, 1966), myristicin showed appreciable toxicity to Mexican bean beetles (*Epilachna varivestis*) and mosquito larvae (*Aedes aegypti*). However, both the route of administration and the dosage level differed from those used in this study. The concentrations of myristicin required to effect mortality by topical application in the earlier studies were 50–100 times greater than those occurring naturally in umbellifer foliage. Synergism of ingested xanthotoxin by myristicin in this study was effected by insecticide–synergist ratios considerably less than those required by myristicin to synergize topically applied synthetic organic insecticides (1:0.01 in this study vs. 1:2–1.0 in previous studies). Interpreting the differences between the results of this and the earlier studies is difficult due to the differences in procedure; however, it is clear from this study that myristicin at levels occurring naturally in umbellifer foliage and fruit is by

itself nontoxic to *H. zea* and even at very low levels is an effective synergist of xanthotoxin.

The fact that there is a significant interaction between xanthotoxin and myristicin on the rate at which caterpillars die, particularly in the absence of main effects, is consistent with the proposed mechanism of synergism by myristicin—that it is a competitive inhibitor of microsomal mixed function oxidases (Wilkinson, 1973). Increasing myristicin concentrations would thereby allow greater amounts of xanthotoxin to circulate unmetabolized, effecting mortality more rapidly than would otherwise be possible in the presence of a fully effective MFO system. The significant interaction between xanthotoxin and myristicin on the duration of the first instar in surviving larvae is also consistent with the proposed mechanism for synergism. Increasing the dosage of xanthotoxin has the same effect on development as does maintaining the same dosage of xanthotoxin and increasing the dosage of myristicin. Again, increasing the quantity of myristicin may, by MFO inhibition, simply increase the effective dose of xanthotoxin circulating unmetabolized in the insect.

Xanthotoxin is a known deterrent (Yajima et al., 1977; Muckensturm et al., 1981; Berenbaum et al., in preparation), and in this experiment significantly more larvae were found off the xanthotoxin diets after 24 h than were off diets lacking xanthotoxin. This deterrency appears to be independent of dosage. However, myristicin had no effect on the proportion of larvae in each treatment off the diet. The synergistic effect of myristicin, then, is presumably at the biochemical and not at the behavioral level.

The findings in this study are significant for at least two reasons. First, they suggest that xanthotoxin and possibly other furanocoumarins are detoxified by midgut MFOs, as suggested by the metabolic work of Ivie et al. (1983), and further implicated by the in vivo synergism of xanthotoxin by piperonyl butoxide (Table 5). More importantly, this study suggests that plants can enhance the effectiveness of small amounts of allelochemicals produced and thereby reduce costs of defense or circumvent the acquisition of resistance to those allelochemicals in herbivores by producing substances that interfere with the detoxification process. If production of secondary compounds entails a metabolic cost (Chew and Rodman, 1979), then the production of synergists can reduce that cost without a concomitant loss in efficacy; 90% mortality of *H. zea*, for example, can be effected by 87.5% less xanthotoxin in the presence of a synergist (Table 1). This reduction, equivalent to 1.75 g/100 g fresh weight of tissue, is brought about by only 100 mg of myristicin/100 g fresh weight of tissue, a substantial savings at least in carbon allocation and possibly energy expenditure.

Many economically important members of the Umbelliferae, such as carrot (*Daucus carota*), parsnip (*Pastinaca sativa*), celery (*Apium graveolens*), parsley *Petroselinum crispum*, dill (*Anethum graveolens*), and fennel (*Foeniculum vulgare*) show variation in MDP levels (Stahl and Kubezcka, 1969; Ashraf et al.,

1979). Selective breeding for MPDs in certain cultivars may well reduce or obviate the need for additional pesticides to effect control, since MDPs may potentiate endogenous toxins to an extent sufficient to reduce insect damage. MDPs may also synergize pesticides applied systemically or topically to crop plants. Myristicin is, in fact, an effective synergist of carbaryl in *Heliothis zea* and other insects (Lichtenstein and Casida, 1963; Lichtenstein, 1966; J. Neal and M. Berenbaum, in preparation). Such natural synergists, which may be widespread among crop plants, may permit considerable economic savings in terms of reduced costs of insecticide contamination of soil and nontarget organisms, as well as reduced selection for insecticide resistance in crop pests.

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APPENDIX 1. DIET INGREDIENTS (FOR 50 CATERPILLARS)

500 ml water
11.9 g agar
61.25 g Bio-Serv Bio-Mix 9249[a] (boll weevil rearing media, modified
Vanderzant, consisting of casein, sucrose, wheat germ, Wesson salts, cholesterol, choline chloride, inositol, and ascorbic acid)
0.55 g cysteine
27.8 g Alphacel
1.1 g methyl-*p*-hydroxybenzoate
2.22 ml 10% formaldehyde
0.13 g streptomycin
2.8 ml 4 M KOH
1.3 g ascorbic acid
1.3 g sorbic acid
5.5 g corn oil

[a]Bio-Serv, Inc., Frenchtown, New Jersey.

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# MULTIPLE ACCEPTORS FOR PHEROMONAL ENANTIOMERS ON SINGLE OLFACTORY CELLS IN THE DOUGLAS-FIR BEETLE, *Dendroctonus pseudotsugae* HOPK. (COLEOPTERA: SCOLYTIDAE)[1,2]

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**Abstract**—Olfactory perception of pheromonal enantiomers by male and female Douglas-fir beetles, *Dendroctonus pseudotsugae* Hopk. (Coleoptera: Scolytidae), was investigated by electrophysiological techniques and behavioral bioassays. Electroantennograms (EAGs) and single-cell responses indicated both sexes to be more responsive to racemic frontalin and the (−)-enantiomer at lower dosages. At higher dosages, little difference was noted in responses to either enantiomer. However, response to the racemic mixture at higher dosages was slightly greater than responses to either enantiomer alone. In laboratory behavioral bioassays, responses to low concentrations of (−)-frontalin and the racemic mixture exceeded response to the (+)-enantiomer alone. At a higher concentration, responses to the racemic mixture or either enantiomer alone did not differ. The results indicate that separate enantiomer-specific acceptors may exist on the same pheromone receptor cell.

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**Key Words**—Olfaction, receptor cell, chirality, perception, neurobiology, pheromone, enantiomer, bark beetle, electrophysiology, Douglas-fir beetle, *Dendroctonus pseudotsugae*, Coleoptera, Scolytidae, (+)-frontalin, (−)-frontalin.

## INTRODUCTION

The chirality of semiochemicals has been found to play an important role in both communication within insect species and insect–host plant interactions (see Silverstein, 1979; Städler, 1974). Enantiomeric specificity is maintained by the insect and its host through both the chirality of pheromone precursors available from the host (Renwick et al., 1976) and enantiomeric specificity of synthetic enzymes (Renwick and Dickens, 1979). In addition, communication of chirality is further facilitated by enantiomer-specific acceptors (receptor sites) on insect olfactory cells (Kafka et al., 1973; Dickens and Payne, 1977; Mustaparta et al., 1980; Payne et al., 1982; Wadhams et al., 1982).

The aggregation pheromone of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopk. (Coleoptera: Scolytidae), is released by females initiating host attack and attracts both sexes for mass attack of a potential host (Rudinsky, 1973; Rudinsky and Ryker, 1977). This pheromone consists of at least seven odorants (Ryker et al., 1979) including: 3,2-MCHone (3-methyl-2-cyclohexenone) (Kinzer et al., 1971); 3,2-MCHol (3-methyl-2-cyclohexenol) (Vité et al., 1972; Rudinsky et al., 1974); frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) (Kinzer et al., 1969; Pitman and Vité, 1970; Rudinsky et al., 1974); verbenone (Rudinsky et al., 1974) and *trans*-verbenol (Rudinsky et al., 1972). However, a mixture of 3,2-MCHone (in low concentrations), 3,2-MCHol, and frontalin was found to be most attractive in field tests (Rudinsky et al., 1974).

The olfactory receptor system of *D. pseudotsugae* for pheromones and host odors has been investigated at both the electroantennogram (EAG) (Dickens et al., 1983) and single-cell levels (Dickens et al., 1984). These investigations revealed that antennal olfactory cells could be classified into four types based on their sensitivity and specificity for the pheromones and host odors tested. Three of the cell types were primarily sensitive to one of the major aggregation pheromone components, i.e., 3,2-MCHone, 3,2-MCHol, or frontalin, while a fourth cell type responded primarily to compounds which were behavioral synergists.

Previous experiments showed racemic frontalin to be active at the antennal receptor (Dickens et al., 1983, 1984) and behavioral levels (Rudinsky et al., 1974). However, frontalin exists in two enantiomorphic forms (Mori, 1975; Stewart et al., 1977) and in other *Dendroctonus* species, specificity occurs in both neural perception (Payne et al., 1982) and behavioral response to individual enantiomers (Wood et al., 1976; Payne et al., 1982).

The purpose of this investigation was to elucidate antennal receptor and behavioral responses of *D. pseudotsugae* to the enantiomers of frontalin using electrophysiological techniques and laboratory bioassays.

## METHODS AND MATERIALS

*Insects.* Adult *D. pseudotsugae* used in this study emerged from naturally infested bolts of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, from the McDonald Forest of Oregon State University, Corvallis, Oregon. Following emergence, beetles were sexed (Jantz and Johnsey, 1964) and maintained on moist filter paper in Petri dishes at 6°C until use within 1–3 weeks.

*Electrophysiology.* EAG and single-cell recording techniques were previously described in detail (Dickens, 1979) and were a modification of earlier techniques (Schneider, 1957; Boeckh, 1962). In brief, EAGs were recorded with Ag–AgCl capillary electrodes filled with 3 M KCl. The recording electrode was inserted into the distal end of the antennal club following prepuncture with a sharpened tungsten needle. The indifferent electrode was implanted in the mouth.

Single-cell recordings were made with 50.8 μm diameter tungsten wire electrolytically sharpened to a tip diameter of ca. 1–2 μm. The recording electrode was positioned under optical control (320×) with a Leitz high-power micromanipulator near the base of one of the three sensory bands encircling the antennal club. The indifferent electrode was implanted in the body of the beetle through the oral cavity.

Electrical signals were amplified 10× by a Bioelectric NF1 preamplifier prior to display on a Tektronix 561B oscilloscope. For single-cell recordings, the signal was further conditioned by a Tektronix 122 low-level preamplifier. Records of electrical activity were made on Polaroid film with a Tektronix 405 oscilloscope camera.

*Chemical Stimuli and Experimental Protocol.* Stimulus compounds and their source and purity are summarized in Table 1. Compounds were prepared as serial dilutions in nanograde pentane and presented as 10 μl samples placed on filter paper (20 × 7 mm) inserted into glass cartridges (75 mm; 5 mm ID) oriented toward the preparation from ca. 1 cm. Stimulus duration was 1–2 sec; air flow was ca. 2 liters/min.

Stimulus dilutions were presented from the lowest to the highest concentration. At least 3 min were allowed between each stimulus, except at higher concentrations, when 5 min were allowed between successive stimuli. These intervals were adequate for complete recovery of both EAG and single-cell activity.

To compare EAGs from different preparations, stimulation with a racemic frontalin standard at 10 μg occurred between every two stimulations with serial

TABLE 1. SOURCE AND PURITY OF STIMULUS COMPOUNDS USED IN ELECTROPHYSIOLOGICAL AND BEHAVIORAL EXPERIMENTS

| Compound | Chemical purity (%) | Optical purity (%) | Source |
|---|---|---|---|
| (±)-Frontalin | 99 | | A[a] |
| (+)-Frontalin | 99 | >98 | B |
| (−)-Frontalin | 99 | >98 | B |
| (±)-Limonene | 99 | | A |

[a] A, Chem. Samp. Co., Columbus, Ohio, B, Dr. K. Mori (Mori, 1975).

dilutions. Responses to intervening test stimuli were represented as a percent of the mean of the nearest two responses to the standard.

For each EAG stimulus, five replicates (i.e., five males and five females) were recorded from both sexes. Single-cell studies included responses of two frontalin cells (one male; one female) and four synergist cells (one male; three females) to both the racemic mixture and the individual frontalin enantiomers (for classification of cell types see Dickens et al., 1984). Each stimulus was presented only once to a particular cell.

*Laboratory Bioassays.* Laboratory behavioral bioassays were conducted using an olfactory walkway described in detail elsewhere (Jantz and Rudinsky, 1965; Kinzer et al., 1971). A complete stop over the test vial was used as the criterion for arrestment. Dilutions of test chemicals used in the bioassay were prepared in 95% ethanol. Each treatment was replicated four times (total of 20 males; five insects per replicate). Means were compared for significant differences using analysis of variance and $F$ statistic (Ostle, 1963).

## RESULTS

*Electrophysiology.* Dosage–response curves constructed from EAGs of male and female *D. pseudotsugae* to the racemate and individual enantiomers of frontalin were similar (Figure 1). For each sex, (−)-frontalin elicited EAGs greater than (+)-frontalin at the lowest dosages; response to the racemate was intermediate. At the highest dosage tested, the greatest response was elicited by the racemic mixture followed by (+)-frontalin then the (−)-enantiomer. The dosage–response curve for racemic frontalin seemed to parallel that of the (−)-enantiomer at the lower dosages. At the higher dosages, a similar parallel relationship was found between the dosage–response curves for the (+)-enantiomer and the racemate.

Results from the single-cell studies were similar to those from EAGs in that

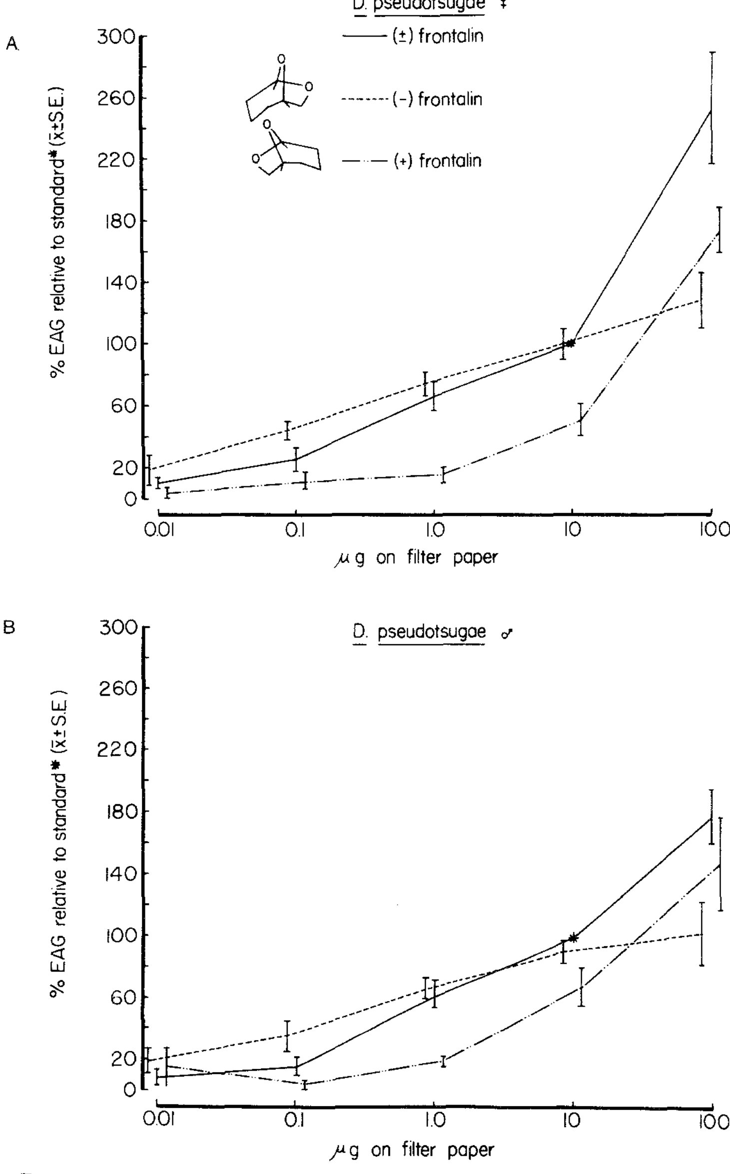


FIG. 1. Dosage–response curves constructed from EAGs of female (A) and male (B) *D. pseudotsugae* to racemic frontalin and its optical antipodes. Each point represents the mean of five replicates. Vertical bars represent standard errors.

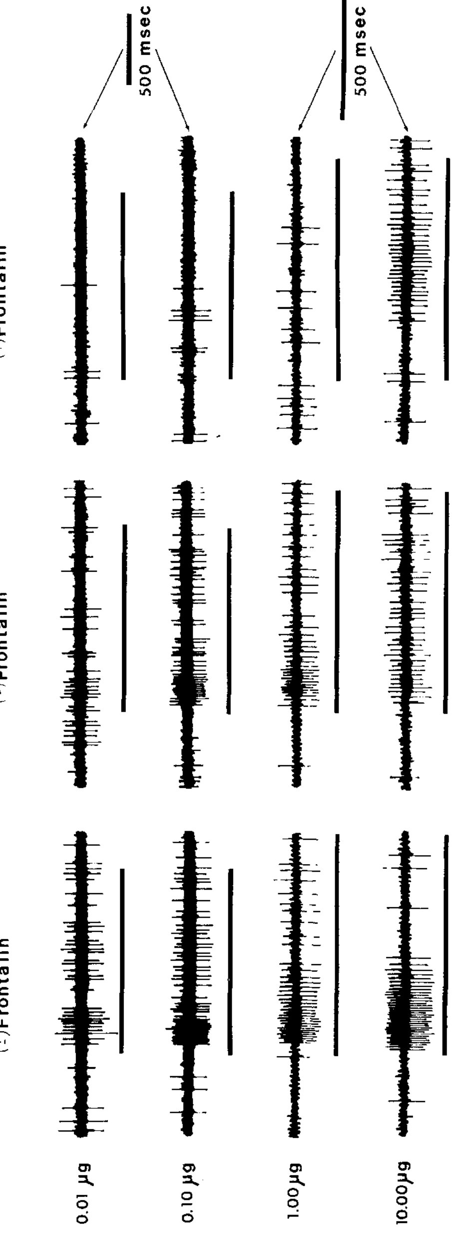


FIG. 2. Response of a frontalin cell to serial dilution of racemic frontalin and its optical antipodes.

at lower dosages racemic frontalin and the (−)-enantiomer elicited considerably more spikes than did the (+)-enantiomer (Figures 2 and 3). However, response to (+)-frontalin at the highest dosage tested was nearly equal to the number of impulses elicited by (−)-frontalin, while response to the racemate exceeded response to either enantiomer alone.

A similar response pattern was observed for synergist cells activated by relatively high dosages of frontalin (Figure 4). For example, a cell primarily sensitive to the host terpene, limonene, was most responsive to racemic frontalin at the 1.0 μg and 10 μg dosages with an intermediate response to the (−)-enantiomer relative to the least active (+)-enantiomer. However, at the highest dosage, i.e., 100 μg, little difference was noted between responses to all three compounds.

*Laboratory Bioassays of Frontalin Enantiomers.* Pedestrian bioassays confirmed results obtained in electrophysiological experiments (Table 2). At the lowest dosage tested, both (−)-frontalin and racemic frontalin arrested a larger number of male *D. pseudotsugae* than did the (+)-enantiomer. However, at the highest dosage tested, little difference was noted between the number of males arrested by any of the three treatments.

## DISCUSSION

The fact that the enantiomers of frontalin stimulated a common single receptor cell is evidence that a single receptor cell can possess acceptors for both enantiomers. Furthermore, greater response at the EAG and single cell levels to racemic frontalin, relative to either enantiomer alone at the highest dosage tested,

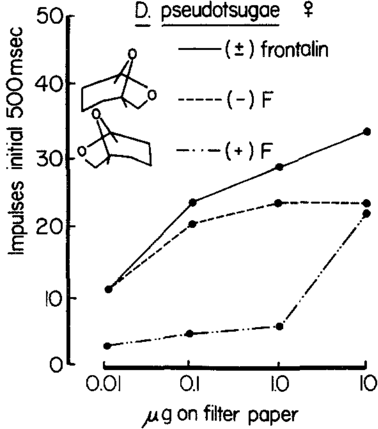


FIG. 3. Dosage–response curves constructed from response of a frontalin cell to racemic frontalin and its optical antipodes.

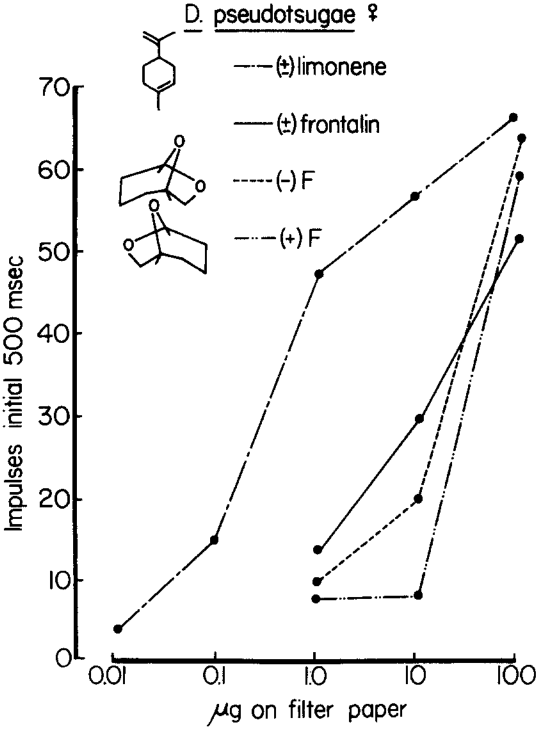


FIG. 4. Dosage–response curves constructed from response of a synergist (limonene) cell to racemic frontalin and its optical antipodes.

TABLE 2. RESPONSE OF WALKING MALE DOUGLAS-FIR BEETLES TO ETHANOL SOLUTIONS OF FRONTALIN ENANTIOMERS

| Frontalin (0.01 μg/μl)[a] | Arrested[b] | Frontalin (0.1 μg/μl) | Arrested |
|---|---|---|---|
| (+)-Frontalin | 0.8 ± 0.42 | (+)-Frontalin | 3.0 ± 0.41 |
| (−)-Frontalin | 3.8 ± 0.48** | (−)-Frontalin | 4.3 ± 0.48 |
| (±)-Frontalin | 4.0 ± 1.08** | (±)-Frontalin | 2.8 ± 0.71 |
| Ethanol | 0.0 | | |

[a] All solutions in 95% ethanol.

[b] Mean ± SE of 20 beetles (four replications). Means significantly different by ANOVA and $F$ statistic, $^{**}P < 0.10$.

suggests the existence of separate specific acceptors for each enantiomer on a single cell.

Existence of separate acceptors for individual enantiomers on the same receptor cell is further substantiated by recordings from synergist cells (Figure 4). At both the 1.0 $\mu$g and 10 $\mu$g dosages, the number of impulses elicited by frontalin exhibited the following order: ($\pm$)-frontalin > ($-$)-frontalin > (+)-frontalin. The greater response to the racemic mixture in this instance would also indicate the existence of both (+)- and ($-$)-enantiomer acceptors on this cell.

In support of the electrophysiological data, laboratory behavioral bioassays revealed a lower threshold for racemic frontalin and the ($-$)-enantiomer (Table 2). However, at a higher dosage, little difference was seen between the responses to either treatment.

These results indicate that separate acceptors for each enantiomer may exist on the same frontalin receptor cell. Furthermore, even in the case of less specialized synergist cells, differential responses to the individual enantiomers were preserved. Thus perception of frontalin enantiomers by *D. pseudotsugae* appears to occur via across-fiber patterning (O'Connell, 1975) elicited by the interaction of individual enantiomers with chiral acceptors on individual cells. A similar mechanism for the perception of frontalin enantiomers was hypothesized for the sourthern pine beetle, *D. frontalis* (Dickens and Payne, 1977; Payne et al., 1982). However, dosage–response data to further support the proposed hypothesis was unavailable for this species.

While an examination of the nerve impulses recorded from the sensitive sensilla suggest that the acceptors sensitive to both (+)- and ($-$)-frontalin are on the same neuron, a remote possibility exists that they may occur on separate neurons. It is possible, although highly unlikely, that the recording electrode was positioned in such a way between two cells, one sensitive to (+)-frontalin, the other sensitive to ($-$)-frontalin, so that the recorded spikes would be similar. However, neither doubling nor short interspike intervals occurred in response to the racemate. Thus we are left to conclude that multiple acceptors may occur on the same cell.

These findings for *Dendroctonus* species are similar to those observed for other insects. Kafka et al. (1973) first observed olfactory discrimination of enantiomers by the migratory locust and honeybee, both in single-cell recordings and behavioral experiments. They explained their observations by hypothesizing the presence of separate acceptors for each enantiomer on the same cell. Similarly, single cells responsive to both optical antipodes of a compound have been reported for other coleopterous species (Wadhams et al., 1982; Hansen, 1983).

These observations are an interesting contrast to the system proposed for discrimination of enantiomers by *Ips* species (Mustaparta et al., 1980). It was hypothesized for *Ips* species that olfactory cells possessed acceptors for only one enantiomer of a pheromone. But, in fact, these authors found individual receptor

cells primarily sensitive to one enantiomer did respond to its optical antipode at a higher dosage. They proposed that since the enantiomers they used were impure (i.e., 92% optically pure), response to the least active enantiomer could be attributed to the presence of its optical antipode. Furthermore, the intermediate response to the racemate was considered to be supportive of their hypothesis. In addition, a response similar to that of the most active enantiomer was obtained from stimulation with the racemate at 2× concentration (i.e., 100% of active enantiomorph). However, as they point out, their hypothesis is contradicted by different saturation levels for each enantiomer on individual cells.

Thus two slightly different mechanisms for the perception of pheromonal enantiomers appear to exist among insects. In *Dendroctonus* species, as in several other insects, individual cells specialized for the perception of an odorant may have varying numbers of acceptors for each enantiomer, with the resulting chiral message being coded across several cells (Kafka, 1973; Dickens and Payne, 1977; Wadhams et al., 1982; Hansen, 1983). As previously reported (Dickens and Payne, 1977; Dickens et al., 1984), and shown here, the occurrence of multiple acceptors on a single olfactory cell is not limited to enantiomers of a single pheromone. In fact, a single cell may possess acceptors for different pheromones and even host odors. Such a system of olfactory perception provides the insect with greater flexibility in coding sensory messages than might be possible via labeled lines. Another mechanism for deciphering pheromonal chirality proposed for *Ips* species involves "labeled lines," whereby acceptors for only one enantiomer occur on each cell (Mustaparta et al., 1980). However, this second mechanism should be considered tentative until pure enantiomers are available for testing.

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# SYNTHESIS OF STEREOISOMERS OF 8-METHYL-2-DECANOL AND ESTERS ATTRACTIVE TO SEVERAL *DIABROTICA* SP.[1]

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**Abstract**—Details of the syntheses of the four stereoisomers of 8-methyl-2-decanol and its propanoate ester are given. The racemic ester, two of its stereoisomers, and one stereoisomer as an acetate are attractive to several species of *Diabrotica*. The key steps in the syntheses involve high-performance liquid chromatograpic resolutions of diastereomers to achieve high configurational enrichment of each site and generation of (*R*)-2-methylbutyric acid by chemical degradation of D-isoleucine.

**Key Words**—Asymmetric synthesis, stereoisomers, sex pheromone, *Diabrotica*, Coleoptera, Chrysomelidae, (*R*)-2-methylbutyric acid, HPLC, diastereomers.

## INTRODUCTION

The sex pheromone emitted by the female western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, was isolated and identified as 8-methyl-2-decanol propanoate (Guss et al., 1982). A nonstereoselective route to this structure was described briefly in conjunction with the identification, and we noted that the synthetic material was essentially as active as the natural product. An outline of the synthesis of two of the four stereoisomers of the parent structure has been reported (Sonnet and Heath, 1982), and an independent synthesis of two of these isomers also has been accomplished (Mori and Watanabe, 1984).

[1]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

A number of field observations have been made using the four stereoisomers of 8-methyl-2-decanol propanoate that indicate widespread use of these compounds, and probably the corresponding acetates, in the sex communications of *Diabrotica* species (Guss et al., 1982, 1983a,b, 1984, 1985). We wish therefore to report the experimental detail for our syntheses of these isomers, as it appears that some considerable benefit will accrue to the biological research through their continued availability.

## METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed with a Varian 2400 instrument using the following columns at various temperatures: OV-1® (0.25 mm ID × 1.5 m), SP-2340 (2.5 mm ID × 20 m), cholesterol parachlorocinnamate (0.25 mm ID × 15 m), cholesterol paranitrocinnamate (0.25 mm ID × 15 m), and UB-II (3.2 mm × 1.5 m). A Varian 3700 instrument was employed with a fused silica column of SE-52 (0.25 mm × 30 m) and a column of 3% OV-17 on 100–120 mesh support (2 mm × 2 m glass). A Hewlett-Packard model 402B instrument was used that was equipped with a glass column of 3% phenyldiethanolamine succinate (PDEAS) on 100–120 mesh support (2 mm × 2 m). GLC peak integration was obtained with a Hewlett-Packard model 3354 laboratory data system. Helium was the carrier gas (linear flow velocity = 18 cm/min) for the capillary columns, and detection was in all cases by flame ionization.

High-performance liquid chromatography (HPLC) was performed with a Waters Associates model 6000 pump and RI detection using solvents as described below and the following columns: analytical work 5 μm LiChrosorb Si-60 (6.3 mm × 25 cm) and preparative work 2–10 μm Biosil-A (25 mm × 25 cm). HPLC was performed also on a DuPont Instruments model 830 chromatograph equipped with a UV detector and a Brownlee Laboratory 6.3-mm × 25-cm column of 5 μm LiChrosorb SI-100. Additional preparative HPLC separations were performed on a Waters Associates model 500 instrument fitted with two silica cartridges.

Optical rotations were measured with a Rudolph Research Autopol III automatic polarimeter. Infrared (IR) spectra were obtained on a Perkin-Elmer model 281 grating spectrometer or on a Perkin-Elmer model 467 spectrophotometer (3% solutions in $CCl_4$). Nuclear magnetic resonance (NMR) data were obtained with a Nicolet 300 MHz FTNMR spectrometer (1% solutions in $CDCl_3$ for $^1H$ spectra), or using deuterochloroform solutions with a Varian model T-60 or JEOL Model FX-90Q spectrometer. Mass spectra were obtained with a Finnegan model 3200 spectrometer operated for CIMS (isobutane) and that was serviced by a Varian model 1400 GLC fitted with an OV-101 column (2.5 mm ID × 1.5 m).

The α-methylbenzylamines were purchased from Hexcel Corp. (Zeeland, Michigan) [the batch of *S* enantiomer we used had been found to be 99.4% S using GLC analysis of amides of methoxytrifluoromethylphenylacetic acid, MTPA (Dale and Mosher, 1973)]. (*R*)-1-(1-Naphthyl)ethylamine was purchased from Norse Laboratories (Santa Barbara, California) and purified by crystallization of the hydrogen *L*-tartrate salt to give material of 99.4% ee (Bergot et al., 1978). The corresponding isocyanate was prepared by reaction of the amine with phosgene (Pirkle and Hoekstra, 1974). D-Isoleucine was purchased from Sigma Chemical Co. (*S*)-2-Methylbutanol (puriss. grade) purchased from Fluka Chemical Co. was shown, by oxidation to 2-methylbutanoic acid and HPLC analysis of the corresponding amide with (*R*)-1-(1-naphthyl)ethylamine, to contain no more than 0.8% of the (*R*)-enantiomer. A sample of this alcohol from Aldrich Chemical Co. contained about 0.5% (*R*). Melting points were obtained on a Fisher-Johns hot-stage apparatus and are uncorrected.

*cis- and trans-1-Bromo-4-methyl-3-hexene, II.* Cyclopropyl methyl ketone (16.8 g, 0.20 mol) was added dropwise to an ethereal solution of ethyl magnesium bromide (Ventron, 68 ml of 2.2 M) at 5–10°C (Julia, 1961). After 1 hr at room temperature, the reaction was worked up by carefully adding aqueous $NH_4Cl$. The solvent layers were separated, and the ethereal layer washed with water and then concentrated to a small volume. The crude product was added in some pentane dropwise to 60 ml of cold 48% HBr, and the resulting mixture was stirred (0–5°C) for 1 hr. The organic product was extracted with additional pentane and washed with water. After drying ($Na_2SO_4$), the pentane layer was concentrated and passed through Brockman neutral alumina (40 g)with pentane (200 ml). The pentane was removed and the product distilled to give 26.1 g (73.7%) of II: bp 76–82°C (30 mm), lit. bp 76–80°C (27 mm) (Julia, 1961); EIMS *m/e* 178, 176 (M).

*1-Bromo-4-methylhexane, III.* Bromide II (20.3 g, 0.115 mol) was hydrogenated over 5% Pt on carbon (0.5 g) in acetic acid (75 ml) at 1 atm and room temperature. After the usual work-up (filtration, water dilution, extraction with pentane), the crude product was distilled to give 16.5 g (80.0%) of III: bp 71–75°C (25 mm); $^1$HNMR δ 0.86 (6H, m, 2 $CH_3$s), 3.39 (2H, t, $J = 7$ Hz, $CH_2\underline{CH_2}Br$); EIMS (M) *m/e* 180, 178 (M).

*Racemic 8-Methyl-3-decyn-2-ol, IV.* The tetrahydropyranyl (THP) ether of 3-butyn-2-ol (bp 80–85°C at 21 mm) (13.8 g, 89.4 mmol) was converted to its lithio derivative in tetrahydrofuran (THF) (80 ml) with butyllithium (BuLi) (37.3 ml of 2.4 M) under nitrogen and at ≤0°C. Bromide III (16.0 g, 89.4 mmol) was added, then hexamethylphosphoric triamide (HMPT) (30 ml), and the resulting mixture was allowed to stir overnight at room temperature. The mixture was diluted with water and extracted with hexane. The crude alkylation product obtained by removing the hexane was dissolved in methanol (100 ml) containing HCl (1.5 ml of 12 N). After 2.5 hr, the solution was diluted with water and extracted with hexane. The dried ($MgSO_4$) organic phase was concentrated, and

the residue was distilled to give 12.4 g (82.7%) of IV: bp 65–66°C (0.02 mm); IR 3500 $cm^{-1}$, broad; [$^1$H] NMR δ 0.89 (6H, m, $CH_3$s), 1.28 ($CH_2$ envelope), 1.49 (3 H, d, $J$ = 6.8 Hz, $C\underline{H}_3CHOH$), 2.18 (2H, m, $CH_2C{\equiv}C$, 5.46 (1H, m, $C\underline{H}OH$) ppm. CIMS *m/e* 169 (M + 1), 151 (M + 1 − $H_2O$).

*Racemic 8-Methyl-2-decanol and its Propanoate, I-Racemic.* Alkynol IV (10.0 g, 60 mmol) was hydrogenated in acetic acid (100 ml) over 5% Pt on carbon (0.5 g). Distillation gave 8.4 g (84%): bp 51–54°C (0.005 mm); IR 3500 $cm^{-1}$ broad; [$^1$H]NMR δ 0.88 (6H, overlapped $CH_3$s), 1.18 (ca. 3H, d, $J$ = 6.8, $C\underline{H}_3CHOH$), 1.27 ($CH_2$ envelope), 3.81 (1H, m, $C\underline{H}OH$) ppm. CIMS *m/e* 173 (M + 1), 155 (M + 1 − $H_2O$).

A solution of racemic 8-methyl-2-decanol (7.7 g, 47.7 mmol) in pyridine (25 ml) was stirred in ice and propionic anhydride (7.1 ml, 55 mmol) was added. The resulting solution was then warmed to 45–50°C for 2 hr and was finally worked up by pouring over cracked ice and dilute HCl, and extracting with hexane. The extract was washed with water and then aqueous $NaHCO_3$ until the washings were slightly alkaline. The extract was dried ($MgSO_4$) and concentrated, and the residue was distilled to give I-racemic, 9.3 g (91.1%): bp 60–65°C (0.005 mm); spectral data as previously published (Guss et al., 1982).

*(2R, 8R, S)-8-Methyl-2-decanol Propanoate, I-(2R, 8R, S).* The racemic alkynol IV (3.0 g, 18.0 mmol) was treated with $R$-α-methoxy-α-trifluoromethylphenyl acetyl chloride (MTPA-acid halide) (1 equiv) in pyridine-$CCl_4$ (30 ml of 1 : 1) with ice-cooling to prepare the corresponding diastereomeric MTPA esters. These were resolved on all of our GLC columns with the $R,R$-diastereomer expected to elute first (Doolittle, unpublished data), while only partial resolution could be effected with the diastereomeric saturated alcohol MTPAs. With the (packed) UB-II column at 160°C: $\alpha$ = 1.08 for alkynol-MTPA esters the ($R,R$)-diastereomer eluted first, however, and separations were much greater using the cap. columns. Thin-layer chromatography (TLC) with 10% EtOAc–hexane on unactivated silica gel plates gave: $R,R$-diastereomer, $R_f$ = 0.76; $R,S$, $R_f$ = 0.69. Preparative HPLC using 1% ether–hexane was employed to purify the diastereomeric MTPA esters: $\alpha$ = 1.17 and $k'_{R,R}$ = 2.5, $k'_{R,S}$ = 2.92; [$^1$H]NMR δ ($R,R$-diastereomer) 0.86 (6H, m, $CH_3$s), 1.49 (3H, d, 6.6 Hz, $CHC\underline{H}_3$), 2.19 (2H, m, $CH_2C{\equiv}C$), 3.60 (3H, s, $OCH_3$), 5.66 (1H, m, OCH), 7.26, and 7.40 (5H, m, aryl H); ($R,S$-diastereomer) the same except 1.56 (3H, d, $J$ = 6.6 Hz, $CHCH_3$). The purified diasteromers were judged to be 97% pure by GLC.

Each alkynol MTPA ester was hydrogenated in acetic acid over 5% Pt on carbon, and the reaction was monitored by GLC. The saturated MTPA derivatives could be partially resolved on the cap. cholesterol paranitrocinnamate column: k $R,R$ = 8.267, $R,S$ = 8.422, $\alpha$ = 1.019, resolution = 0.5. These diastereomers were not resolved by HPLC.

Saponification of the esters was conducted in 1 : 1 4 N KOH–methanol under reflux overnight. The alcohols (V) were obtained from the cooled reaction mixtures after dilution with water and extraction with hexane. Each alcohol was

then converted to its propanoate ester as described above. Appropriate chromatographic and spectral comparisons were made with racemic material. Overall yields of I from its MTPA ester that was 94% *E,E* at the 2 carbon were 90–95%.

*(R)- and (S)-2-Methylbutanoic Acid.* The (*S*) acid was prepared by oxidation of (*S*)-2-methyl-1-butanol using Jones reagent (Fieser and Fieser, 1967). The distilled acid was converted for analytical purposes to an amide of (*S*)-$\alpha$-methylbenzylamine and shown to be 99.5% (*S*) (GLC) (Sonnet and Heath, 1982). The (*R*)- and (*S*) acids also could be obtained by preparative HPLC separation of diastereomeric amides of this same amine. The amides were prepared by conversion of the racemic acid (13.7 g, 125 mmol) to the acid halide with $SOCl_2$ (10 ml, 139 mmol) and dimethylformamide (0.8 ml, 10 mmol) in anhydrous ether (75 ml) at room temperature for 2 hr. The ethereal solution was purged of HCl by bubbling $N_2$ through it. The solution was then added dropwise to a stirred, ice-cooled solution of (*S*)-$\alpha$-methylbenzylamine (15.3 ml, 120 mmol) and triethylamine (21 ml, 150 mmol) in ether (120 ml). The amide was obtained by washing the reaction mixture with dilute HCl, then water, drying ($MgSO_4$), and removing the solvent. The diastereomers were purified by preparative HPLC using THF–EtOAc–hexane (1:2:7), $\alpha \sim 3$.

The order of elution is *R,S*-diastereomer first (Helmchen et al., 1977). Pertinent data are: *R,S*-diastereomer mp 98–100°C (aq. ethanol); IR = 3460, 1655 $cm^{-1}$; [$^1$H]NMR $\delta$ 0.88 (3H, t, $J$ = 7.0 Hz, C$\underline{H}_3$CH$_2$), 1.14 (3H, d, $J$ = 6.9 Hz, C$\underline{H}_3$CHC=O), 1.49 (3H, d, $J$ = 6.9 Hz, C$\underline{H}_3$CHN), 1.4–2.2 (other CH, m), 5.15 (1H, CH$_3$C$\underline{H}$C=O), 7.3 (5H, m, aryl H); CIMS *m/e* 206 (M + 1), 105 ($C_6H_5CHCH_3$), 102 ($C_2H_5CHCH_3C(OH){=}NH_2$); *S,S*-diastereomer mp 86–87°C (aq. ethanol); other spectral data indistinguishable. The diastereomer incorporating the *R*-acid was *N*-hydroxyethylated (Sonnet, 1982) and then hydrolyzed in 1 N HCl under reflux (10 ml: 1 mmol amide) for 2 hr. The mixture was cooled and extracted with ether. The ethereal extract was washed with brine and then dissolved into cold 1.25 N NaOH. The aqueous phase was washed with ether, acidified, and extracted with ether. The ether layer was dried ($MgSO_4$) and concentrated, and the residue was distilled to give (*R*)-2-methylbutanoic acid in 58% yield: bp 70–76°C (30 mm, short path). The acid was analyzed as its $\alpha$-methylbenzamide by GLC and was 97% *R* (94% *E,E*).

*THP Ether of 6-Methoxy-3-hexyne-2-ol, VI.* The THP ether of 3-butyn-2-ol (15.4 g, 0.10 mol) was converted to its lithio derivative in THF (100 ml as described above). The solution was kept under $N_2$ and at $\leq$0°C as ethylene oxide (5.0 ml, 0.10 mol) and HMPT (18 ml) were sequentially injected. The resulting mixture was stirred overnight at room temperature and then worked up by dilution with water and extraction with ether. The extract was dried ($MgSO_4$) and concentrated, then added dropwise (THF rinse) to a slurry of hexane-washed sodium hydride (7.2 g, ca. 0.15 mol) in THF (40 ml), under $N_2$ and with ice-cooling. The mixture was stirred at room temperature for 1 hr.

Methyl iodide (6.3 ml, 0.10 mol) was added dropwise (ice-cooling) and the mixture stirred at room temperature for 2 hr. The product was obtained by diluting the mixture with water and extracting with hexane. The extract was dried ($Na_2SO_4$) and concentrated, and the residue was distilled to give VI (racemic) 12.9 g (60.8%): bp 140–147°C (20 mm); [$^1$H]NMR: 1.40, 1.43 (diastereomeric $\underline{H}_3$CH d, $J$ = 6.7 Hz), 2.49 (2H, m, $CH_2C{\equiv}C$), 3.37 (3H, s, $CH_3O$), 3.49 ppm (4H, m, 2 $CH_2O$s).

*1-Methoxy-3-hexyn-5-ol Propanoate, VII.* Compound VI (15.0 g, 71.4 mmol) and propanoyl chloride (9.3 ml, 107 mmol) were dissolved in propanoic acid (80 ml) and warmed at 35–40°C overnight. The mixture was diluted with water and extracted with hexane. The organic extract was washed with aq. $NaHCO_3$ until the washing was alkaline. The hexane was dried ($MgSO_4$) and concentrated, and the residue was distilled to give VII, 11.2 g (84.8%): bp 121–123°C (20 mm); [$^1$H]NMR: 1.14 (3H, t, $J$ = 7.2 Hz, $C\underline{H}_3CH_2$), 1.45 (3H, d, $J$ = 6.7 Hz, $C\underline{H}_3CH$), 2.34 (2H, q, $J$ = 7.5 Hz, $O{=}CCH_2$), 2.48 (2H, m, $CH_2C{\equiv}C$), 3.37 (3H, s, $CH_3O$), 3.49 (2H, t, $J$ = 7.0 Hz, $CH_2O$), 5.46 (1H, m, CHO) ppm.

*1-Methoxyhexan-5-ol Propanoate, VIII.* The alkyne VII (11.2 g, 60.9 mmol) was hydrogenated over 5% Pd on carbon (0.5 g) in propanoic acid (40 ml) and worked up in the usual manner. The crude product was distilled to give VIII (10.0 g, 90%): bp 109–113°C (21 mm) with foaming; IR 1740 $cm^{-1}$ CIMS *m/e* 189 (M + 1).

*1-Iodo-5-hexanol Propanoate, IX.* Trimethylsilyl chloride (12.3 ml, 96.6 mmol) was injected into a stirred mixture of NaI (24.2 g, 161 mmol) in acetonitrile (100 ml) under $N_2$ and cooled in ice. After 0.25 hr, VIII (6.06 g, 32.2 mmol) was added. The resulting mixture was stirred at room temperature for 5 hr after which time GLC indicated completion of reaction. The mixture was worked up by diluting it with water and extracting with hexane. The extract was dried ($MgSO_4$) and concentrated; the residue was distilled to give IX (5.48 g, 60%): bp 69–70°C (0.01 mm); IR 1740 $cm^{-1}$; [$^1$H]NMR $\delta$ 1.14 (3H, t, $J$ = 7.5 Hz, $C\underline{H}_3CH_2$), 1.21 (3H, d, $J$ = 6.2 Hz, $C\underline{H}_3CH{-}O$), 2.32 (2H, q, $J$ = 7.5 Hz, $CH_3C\underline{H}_2C{=}O$), 3.19 (2H, t, $J$ = 6.9 Hz, $IC\underline{H}_2CH_2$), 4.91 ppm (1H, m, $\underline{H}C{-}O$).

*(S)-1-Chloro-2-methylbutane, X.* To (*S*)-2-methylbutanol (19.8 g, 0.225 mol) was added gradually with ice-bath cooling 17.2 ml of thionyl chloride (0.24 mol) at a rate such that the temperature remained below 20°C. Tri-*n*-butylamine (0.5 ml) was added and the solution was heated at 90°C. After gas evolution had ceased, the crude product was washed with sodium bicarbonate solution and distilled to give the chloride (12.0 g, 50%): bp 90–99°C, $[\alpha]_D^{23}$ = +1.383° (reported $[\alpha]_D^{25}$ = +1.33°, Brown and Groot, 1942).

*(2*R,S, *8S)-8-Methyl-2-decyl Propanoate, I-(*2R,S,*8S).* A chloromagnesium derivative was prepared from (*S*)-1-chloro-2-methylbutane, X (2.13 g, 20 mmol), and magnesium (0.53 g) in tetrahydrofuran (THF, 8 ml). To a 6.25-ml aliquot of the resulting solution at −40°C was added gradually 12.5 ml of a 1

M solution of lithium chlorocyanocuprate in THF (Carney, 1974). 6-Iodo-2-hexylpropanoate, IX (2.84 g, 10 mmol), was added, and the mixture was allowed to warm slowly to 0°C. After 15 hr at 0–5°C, the mixture was diluted with water and hexane, filtered, and washed with aqueous sodium cyanide. Removal of solvent and chromatography on silica (50 g) with 5% ether in hexane gave the ester I (1.8 g, 79%). GLC analysis indicated 99.4% purity.

*(8S)-8-Methyl-2-decanone, XIII-(8S).* The chloromagnesium derivative was prepared from (*S*)-1-chloro-2-methylbutane and magnesium in THF as described above. To 30 ml of this solution (2 M, 60 mmol) at 0°C was added THF (5 ml) and 1 ml of a 1 M solution of lithium bromide and cuprous bromide (1 : 1) in THF. To this mixture was added the bromoketal, XI (11.15 g, 50 mmol), at a rate such that the temperature did not exceed 0°C. After 14 hr at 0°C, the mixture was diluted with hexane and washed with aqueous ammonium chloride. Removal of solvent gave the crude ketal, XII (11.0 g). This was dissolved in acetone (100 ml), and 2 N sulfuric acid (10 ml) was added. After 6 hr at 23°C, the mixture was worked up by adding aqueous sodium bicarbonate and hexane. Chromatography of the crude product on silica (60 g) eluted with hexane and 8% ether in hexane gave the ketone, XIII-(8*S*) (8.3 g, 98% yield), $[\alpha]_D^{22}$ = +7.764° neat, $d$ = 0.8369); [$^1$H]NMR $\delta$ 0.86 (6H, m, $CH_3$s), 1.27 (11H, $CH_2$ envelope), 2.13 (3H, s, C-1 $CH_3$), 2.42 ppm (2H, t, $J$ = 7.5 Hz, C-3 $CH_2$).

*(2R, S, 8S)-8-Methyl-2-decanol, V-(2R, S, 8S).* To a solution of the ketone XIII-(8*S*) (8.1 g, 48 mmol) in ethanol (20 ml) was added sodium borohydride (0.91 g, 24 mmol). After 1 hr at 23°C, the solution was diluted with aqueous sodium chloride and extracted with hexane. Removal of solvent gave 8.15 g of the alcohol V. This crude product was used directly in the following reaction.

*(8S)-8-Methyl-2-decyl (R-1-(1-naphthyl)ethylcarbamate, XIV-(2R, S, 8S).* To a solution of the (8*S*)-alcohol V-(2*R*, *S*, 8*S*) (5.16 g, 30 mmol) in toluene (20 ml) was added (*R*)-1-(1-naphthyl)ethyl isocyanate (7.4 g, 38 mmol) and triethylamine (0.1 ml).The solution was heated under reflux for 20 hr, then was stirred with water and extracted with hexane. Removal of solvent and chromatography on silica (60 g) eluted with 15% ether in hexane gave a mixture of diastereomeric carbamates (10.1 g, 91.2%). This mixture was separated by preparative HPLC eluting with 7% ethyl acetate in hexane to give the following isomers, in order of elution: XIV-(2*S*, 8*S*): 4.30 g, mp 77–78°C; $[\alpha]_D^{22}$ = +22.78° ($c$ = 8.4, $CHCl_3$); [$^1$H]NMR $\delta$ 0.85 (6H, m, C-8, −10 $CH_3$), 1.20 (16H, m $CH_2$s, C-8 CH, C-1 $CH_3$), 1.62 (3H, d, $J$ = 7 Hz, NCH-$\underline{CH_3}$), 4.88 (2H, m, O-CH, NH), 5.60 (1H, m, NCH), 7.3–8.3 ppm (7H, m, aromatic). Analytical HPLC showed no contamination by the (2*R*, 8*S*) isomer. XIV-(2*R*, 8*S*): 4.49 g, $[\alpha]_D^{22}$ = −8.03° ($c$ = 5.3, $CHCl_3$). NMR spectral data were essentially identical to those of the (2*S*) isomer.

*(2S, 8S)-8-Methyl-2-decanol, V-(2S, 8S).* To a solution of the (2*S*, 8*S*)-carbamate XIV (4.03 g, 10.9 mmol) in THF (35 ml) was added lithium aluminum hydride (1.0 g) in portions. The mixture was heated at reflux for 1 hr, then

hexane (25 ml) was added followed by cautious addition of water (5 ml). After filtration, the solution was sequentially washed with 1 N hydrochloric acid and sodium bicarbonate, then was concentrated under vacuum and chromatographed on silica (50 g) eluted with 5 and 10% ether in hexane to give the (2*S*, 8*S*)-alcohol (1.84 g, 98%); $[\alpha]_D^{21} = +15.782°$ (neat, $d = 0.8307$); [$^1$H]NMR $\delta$ 0.86 (6H, m, C-8, −10 $CH_3$s), 1.17 (3H, d, $J = 6$ Hz, C-1 $CH_3$), 1.22 (13H, m, $CH_2$s, C-8 CH), 2.39 (1H, s, OH), 7.31 ppm (1H, m, C-2 CH). This alcohol was analyzed for epimeric composition at C-2 by reconversion to the (*R*)-1-(1-naphthyl)ethylcarbamate and HPLC: 0.39% (2*R*). An (*R*)-MTPA ester was prepared using the corresponding acid chloride in pyridine: [$^1$H] NMR $\delta$ 1.331 ppm (3H, d, $J = 6$ Hz, $OCH-CH_3$).

*(2R, 8S)-8-Methyl-2-decanol, V-(2R, 8S).* Reduction of the (2*R*, 8*S*)-carbamate XIV (4.09 g, 11.1 mmol) under the conditions used for the (2*S*, 8*S*) isomer, followed by identical chromatographic purification, gave the (2*R*, 8*S*)-alcohol (1.90 g, 99%), $[\alpha]_D^{22} = +0.280°$ (neat, $d = 0.8223$). The NMR spectrum was identical to that of the (2*S*, 8*S*) isomer. The alcohol was analyzed for epimeric composition at C-2 by reconversion to the (*R*)-1-(1-naphthyl)ethyl carbamate and HPLC: 0.9% (2*S*). The (*R*)-MTPA ester was prepared using the corresponding acid chloride in pyridine: [$^1$H]NMR $\delta$ 1.247 ppm (3H, d, $J = 6$ Hz, $OCH-CH_3$).

*(2S, 8S)-8-Methyl-2-decyl Propanoate, I-(2S, 8S).* A solution of the (2*S*, 8*S*)-alcohol V (1.62 g, 9.42 mmol) and propanoic anhydride (1.5 ml) in pyridine (1.4 ml) was heated at 40°C for 13 hr, then was stirred with water, extracted with hexane, and washed with dilute sulfuric acid and sodium bicarbonate. Chromatography on silica (15 g) eluted with 5% ether in hexane gave the (2*S*, 8*S*)-propanoate (2.13 g, 99%), $[\alpha]_D^{25} = +8.5972°$ ($c = 12.7$, $CHCl_3$). GLC analysis indicated 99.3% purity.

*(2R,8S)-8-Methyl-2-decanol Propanoate, I-(2R, 8S).* The (2*R*, 8*S*)-alcohol V (1.56 g, 9.07 mmol) was esterified with propanoic anhydride and purified as described for the (2*S*, 8*R*) isomer to give the (2*R*, 8*S*)-propanoate (2.07 g, quantitative), $[\alpha]_D^{26} = +4.054°$ ($c = 21$, $CHCl_3$). GLC analysis indicated 99.1% purity.

*(R)-2-Methylbutyl p-Toluenesulfonate, XV.* D-Isoleucine (10.0 g, 76.2 mmol) was added to a solution of sulfuric acid (4.6 g) in water (50 ml). A solution of sodium nitrite (6.3 g, 92 mmol) in 30 ml of water was added over 30 min at 10°C. Water was evaporated under vacuum, the residue was extracted with ether, and solvent was removed to give crude 2-hydroxy-3-methylpentanoic acid (7.5 g, 75%). A solution of this acid in water (70 ml) with sulfuric acid (16.7 g) was cooled in an ice bath and potassium permanganate (15.8 g) was added. Some of the aldehyde intermediate apparently volatilized in the subsequent exothermic reaction. Sulfur dioxide was passed over the mixture until it became clear, then the solution was extracted with ether. Removal of solvent gave crude (*R*)-2-methylbutanoic acid (2.1 g, 36%).

Reaction of an aliquot of this acid with thionyl chloride, followed by excess (*R*)-1-(1-naphthyl)ethylamine in pyridine gave the corresponding amide, shown by HPLC to contain no more than 1.9% of the ($R_{ac}$, *S*) isomer by comparison with an authentic sample (see above). To a solution of LAH (2.3 g) in ether (40 ml) was added a solution of the crude methylbutanoic acid (2.0 g) in ether (15 ml). The mixture was heated at reflux for 14 hr, then water (10 ml) was slowly added, solids were removed by filtration, and the solvent was removed by distillation to give crude (*R*)-2-methylbutanol (1.3 g, 75%). To the crude alcohol was added pyridine (15 ml) and *p*-toluenesulfonyl chloride (4.23 g, 22 mmol). After 18 hr at 0°C, water was added and the mixture was extracted, after 15 min, with hexane. Removal of the solvent under vacuum gave the ester XV (3.5 g, 98%), $[\alpha]_D^{22} = -3.396°$ (neat, $d$ = 1.1093).

*(2*R, S, *8*R*)-8-Methyl-2-decanol, V-(2*R, S, *8*R*).* A bromomagnesium derivative was prepared by adding the bromoketal XI (5.58 g, 25.0 mmol) to magnesium (1.0 g) in THF (20 ml). The reaction was initiated by adding iodine and iodomethane (25 μl). This solution was added slowly at −15°C to a solution of the tosylate XV (3.5 g, 14.5 mmol) in THF (5 ml) to which 1 ml of a 1 M solution of lithium bromide and cuprous bromide (1 : 1) had been added. The mixture was held at −5°C for 1 hr and at 0–5°C for 3 hr, then was diluted with hexane and extracted with aqueous ammonium chloride. Solvent was removed, and the residue was chromatographed on silica (30 g), eluted with 5% ether in hexane to give the crude ketal XII-8*R*) (3.2 g). To a solution of the ketal in acetone (20 ml) was added 2 N sulfuric acid (4 ml). After 6 hr at 23°C, the solution was worked up with aqueous sodium bicarbonate and hexane to give the crude ketone XIII-(8*R*) (2.3 g) $[\alpha]_D^{24} = -5.499°$. To a solution of this ketone in ethanol (10 ml) was added sodium borohydride (0.51 g). After 10 hr at 23°C, the solution was diluted with water and extracted with hexane. Removal of solvent and chromatography of the residue on silica (30 g) eluted with ether in hexane gave the alcohol V-(2*R*, *S*, 8*R*) (2.0 g, 86% yield overall).

*(8*R*)-8-Methyl-2-decanol (*R*)-1-(1-naphthyl)ethylcarbamates, XIV-(2*S, *8*R*) and -(2*R, *8*R*).* To the alcohol V-(2*R*, *S*, 8*R*) (2.0 g, 11.6 mmol) in toluene (10 ml) was added (*R*)-1-(1-naphthyl)ethyl isocyanate (2.97 g, 15 mmol) and triethylamine (0.02 ml). The solution was heated under reflux for 36 hr, then was stirred with water and extracted with hexane. The crude product was chromatographed on silica (40 g) and eluted with ether in hexane to give the diastereomeric mixture of carbamates (4.35 g, quantitative). This mixture was separated by preparative HPLC eluting with 7% ethyl acetate in hexane to give the two diastereomers (in order of elution): XIV-(2*S*, 8*R*): 1.64 g, $[\alpha]_D^{24} = +15.82°$ ($c$ = 6.5, $CHCl_3$). Analytical HPLC showed contamination by 0.4% of the other diastereomer. XIV-(2*R*, 8*R*): 1.59 g, $[\alpha]_D^{24} = -10.30°$ ($c$ = 7.4, $CHCl_3$). Analytical HPLC showed no diastereomeric contamination. The NMR spectra of these compounds were essentially identical to those of the (8*S*)-diastereomers.

*(2*S, *8*R*)-8-Methyl-2-decanol, V-(2*S, *8*R*).* The carbamate XIV-(2*S*, 8*R*)

(1.59 g, 4.32 mmol) was added to a solution of LAH (0.5 g) in THF (15 ml) and heated under reflux for 3 hr, then water was cautiously added, the mixture diluted with hexane, and washed with dilute hydrochloric acid. After removal of solvent, the residue was chromatographed on silica (40 g) eluted with ether in hexane to give the alcohol V-(2*S*, 8*R*) (0.64 g, 86% yield), $[\alpha]_D^{21} = 0.925°$ ($c = 12$, $CHCl_3$). GLC analysis indicated 99.4% purity. Reconversion of an aliquot of the alcohol to its (*R*)-1-(1-naphthyl)ethyl carbamate and analysis by capillary GLC showed 0.4% contamination by the (2*R*, 8*R*) isomer.

*(2R, 8R)-8-Methyl-2-decanol, V-(2R, 8R).* Reduction of the carbamate XIV-(2*R*, 8*R*) (1.59 g, 4.32 mmol) under the same conditions used for V-(2*S*, 8*R*) gave, after chromatographic purification, the alcohol V-(2*R*, 8*R*) (0.62 g, 84%), $[\alpha]_D^{22} = -13.86°$ ($c = 12.5$, $CHCl_3$). GLC analysis indicated 96.7% purity. Reconversion of an aliquot to the (*R*)-1(1-naphthyl)ethyl carbamate and analysis by capillary GLC showed 0.8% contamination by the (2*S*, 8*R*) isomer.

*(2S, 8R)-8-Methyl-2-decanol propanoate, I-(2S, 8R).* The alcohol V-(2*S*, 8*R*) (0.62 g, 3.6 mmol) was esterified with propanoic anhydride (1.0 ml) in pyridine (1.0 ml) as described for the (2*S*, 8*S*) isomer. Chromatography of the crude product on silica (15 g) eluted with 5% ether in hexane gave I-(2*S*, 8*R*) (0.80 g, 97%), $[\alpha]_D^{24} = -3.77°$ ($c = 15$, $CHCl_3$). GLC analysis indicated 98.3% purity.

*(2R, 8R)-8-Methyl-2-decyl propanoate, I-(2R, 8R).* The alcohol V-(2*R*, 8*R*) (0.62 g, 3.6 mmol) was esterified and purified as described for the (2*S*, 8*R*) isomer to give the propanoate I-(2*R*, 8*R*) (0.83 g, quantitative), $[\alpha]_D^{26} = -7.967°$ ($c = 15$, $CHCl_3$). GLC analysis indicated 95.3% purity.

## RESULTS AND DISCUSSION

A number of closely related species of *Diabrotica* responded to the racemic parent structure, 8-methyl-2-decanol propanoate, or preferentially to its stereoisomers. The configuration of the 8-carbon, the hydrocarbon center, has thus far always been the (*R*) configuration (Figure 1) in the biologically active isomers. In addition, where inhibition of activity has been observed, the most ef-

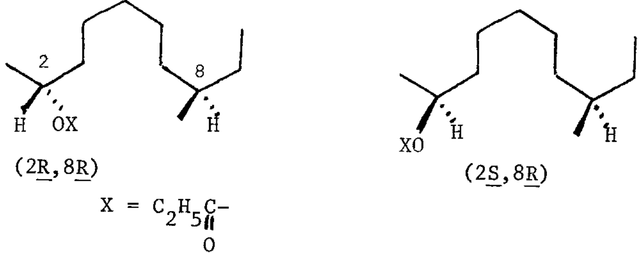


FIG. 1. Biologically active stereoisomers of 8-methyl-2-decanol propanoate.

fective isomer is also the one with (*R*) configuration at the 8-carbon. This center is evidently obliged to be (*R*) for rootworm perception; the stereochemical requirement of the hydrocarbon center means that synthetics may be racemic at that site without affecting insect behavioral response.

In contrast, the 2-carbon (ester site) is apparently species differentiating, and synthetic chemistry must be geared to the biological field result desired. Specifically, the racemic propanoate could be used to monitor for western corn rootworm (WCR), Mexican corn rootworm (MCR), *Diabrotica virgifera zeae* Krysan & Smith, and (although not a pest) *D. porracea* Harold. The isomer racemic at the 8-carbon but with (2*R*) configuration would attract WCR, MCR, and the northern corn rootworm, *D. barberi* Smith, while the corresponding mixture with (2*S*) configuration would attract WCR, MCR, *D. porracea*, *D. longicornis barberi* Smith & Lawrence, and (as the acetate) *D. cristata* Harris. Further studies with individual stereoisomers and selected mixtures of 8-methyl-2-decanol esters should be a rich source of biological research.

Initially we developed a route that would lead to an acetylenic carbinol, e.g., IV (Figure 2) with the hydrocarbon center racemic. The unsaturation was to serve two purposes, namely: (1) to obtain good chromatographic resolution of diastereometric alcohol derivatives, and (2) to examine asymmetric chemical reduction of the corresponding alkynone and kinetic resolution by enzymatic

$C_2H_5MgBr$ → [OH] → HBr → II (Br) → $H_2$,Pt/C → III (Br)

III + LiC≡CCHCH$_3$ (OTHP) → $H_3O^+$ → IV (OH) → 1) $H_2$,Pd/C 2) $(C_2H_5CO)_2O$ → I-racemic

IV → 1) MTPA 2) Prep HPLC 3) $H_2$,Pd/C 4) KOH, MeOH → V

V - 2R,8(R,S)
V - 2S,8(R,S)

→ $(C_2H_5CO)_2O$ →

I - 2R, (8R,S) 94% ee
I - 2S, (8R,S) 94% ee

FIG. 2. Synthesis of racemic (2*R*, 8*R*, *S*)- and (2*S*, 8*R*, *S*)-8-methyl-2-decanol propanoate.

methods of the corresponding acetates/propanoates. The former item is discussed here both in terms of synthesis and analysis; the latter is still under investigation.

Methyl cyclopropyl ketone was allowed to react with the ethyl Grignard reagent and the resulting intermediate carbinol rearranged with HBR to give the homoallylic bromide II (Julia, 1961). Hydrogenation of the alkene using Pt on carbon in acetic acid provided the saturated bromide III. The tetrahydropyranyl ether (THP) of 3-butyn-2-ol was converted to the lithium alkynylide and allowed to react with the bromide III. Acid hydrolysis yielded the propargyl alcohol IV that could be hydrogenated and then esterified in pyridine with propanoic anhydride to produce the racemic WCR pheromone structure, I-racemic.

The propargyl alcohol IV was esterified with the acid halide of $\alpha$-methoxy-$\alpha$-trifluoromethylphenyl acetic acid (MTPA), Mosher's acid (Dale and Mosher, 1973; Sullivan et al., 1973). This derivatizing technique has been employed conventionally to effect enantiomer resolutions. The elution order on silica gel HPLC for alkynols using (*R*)-MTPA is expected to be (*R*)-carbinol first, and (*S*)-carbinol second (Doolittle, unpublished data). Elution order is generally the same on GLC phases. The [$^1$H]NMR shifts of the carbinol methyl were 1.49 (*R*, *R*)-diastereomer) and 1.56 (*R*, *S*) in keeping with the correlations based on configuration originally published (Dale and Mosher, 1973; Sullivan et al., 1973).

The propargyl derivatives were obtained by preparative HPLC and reduced to the saturated MTPA analogs. Independent treatment of the purified and reduced esters with methanolic KOH provided 2*R*, 8*R*, *S* and 2*S*, 8*R*, *S* carbinols that were then propanoylated to give the corresponding I. Each of these materials had 94% *E*, *E* (97 : 3) at the 2-carbon.

We next turned our attention to the synthesis of the four stereoisomers themselves choosing to use (*R*)- and (*S*)-2-methyl-1-butanol as chiral building blocks. The (*S*) enantiomer of 2-methyl-1-butanol is commercially available as a product of fermentation. Analysis of samples by oxidation to the corresponding acid and conversion to the amide with (*S*)-1-phenylethylamine or (*R*)-1-(1-naphthyl)ethylamine and chromatography indicated 99.2–99.5% (*S*) configuration. Several commercial samples of this alcohol were found to be contaminated with 3-methyl-1-butanol.

The (*R*) enantiomer is available by reduction of the corresponding acid, which was prepared in two independent ways (Figure 3). Racemic 2-methylbutoanic acid was converted to a mixture of diastereomeric amides with (*S*)-1-phenylethylamine. Preparative HPLC was followed by labilization of the purified amides to hydrolysis (Sonnet, 1982). The amides were *N*-hydroxyethylated and treated briefly with refluxing 1 *N* HCl. The recovered acids were then distilled and analyzed for configurational purity by reconversion to amides. The (*R*) acid so obtained had 94% *E*, *E*. This procedure compares favorably with that of Carlini et al. (1973) whereby recrystallization of the salt of racemic 2-methylbutanoic acid with (*S*)-1-phenylethylamine produced only 91.4% optically pure

FIG. 3. Preparation of (*R*)-2-methylbutyric acid.

(*R*) acid in 9% yield after 12 recrystallizations. Unfortunately, attempted fractional crystallization by us of the corresponding amide was not very successful either.

Alternatively, the (*R*) acid was more conveniently prepared by oxidative degradation of *D*-isoleucine, which is commercially available. Nitrous acid deamination of this amino acid followed by oxidation with acidic potassium permanganate gave (*R*) acid of 96.2% *E*, *E* (Greenstein and Winitz, 1961). Reduction of the acid with lithium aluminum hydride gave (*R*)-2-methyl-1-butanol, which was converted to the tosylate ester for use in subsequent reactions.

The other half of the molecule was prepared by alkylating the lithio derivative of the THP of 3-butyn-2-ol with ethylene oxide (Figure 4). The crude alkynediol mono-THP was O-methylated to give VI. Treatment with propanoyl chloride gave the secondary propanoate ester VII, which was hydrogenated to VIII and then converted to an iodide IX using trimethylsilyl iodide (TMSI) (Schmidt, 1981).

The two chiral segments of the molecule were coupled through alkylation of a magnesium alkylcyanocuprate intermediate. Conversion of (*S*)-2-methyl-1-butanol to the chloride X followed by metallation with magnesium and reaction at −40° with 1 equiv of lithium chlorocyanocuprate (Carney, 1974) gave the alkylcyanocuprate reagent. Addition of the iodo ester IX gave I-(2*R*, *S*, 8*S*) in good yield. In contrast to the copper-catalyzed Gridnard reagent (Tamura and Kochi, 1972), the alkylcyanocuprate does not attack the propanoate ester group.

FIG. 4. Synthesis of (2*R*, *S*, 8*S*)-8-methyl-2-decanol propanoate.

The preparation of the (8*S*) isomers of I on larger scale, a modified scheme was employed (Figure 5). The Gridnard reagent derived from X was alkylated with the readily available bromoketal XI under copper (I) catalysis. Hydrolysis of the resulting ketal XII followed by reduction gave the alcohol V-(2*R*, *S*, 8*S*). Reaction of this alcohol with (*R*)-1-(2-naphthyl)ethyl isocyanate gave a mixture of diastereomeric carbamates XIV-(2*R*, *S*, 8*S*) that could be separated by preparative HPLC with the (2*S*) isomer eluting first (Pirkle and Hoekstra, 1974; Pirkle and Hauske, 1974). This "anomalous" elution order has been reported for another naphthylethyl carbamate of an alkyl methyl carbinol (Pirkle et al., 1979). The purified carbamate diastereomers were reductively cleaved to give the respective alcohols V-(2*S*, 8*S*) and V-(2*R*, 8*S*). The absolute configurations at C-2 were assigned by comparing the specific rotations of the two alcohols (Mills and Klyne, 1954) and by comparing the [$^{1}$H]NMR chemical shifts of the C-1 methyl groups in the corresponding (*R*)-MTPA esters (Dale and Mosher, 1973).

The (8*R*) isomers were prepared in a similar manner, except that the direction of carbon-carbon bond formation was reversed. Metallation of the bromoketal XI with magnesium and alkylation by the tosylate XV under copper (I) catalysis gave the ketal XII-(8*R*). An identical reaction and purification sequence gave the individual alcohol isomers V-(2*S*, 8*R*) and V-(2*R*, 8*R*).

The configurational purity at C-2 of each of the four alcohols V was determined by reconversion to the (*R*)-1-(1-naphthyl)ethyl carbamate and analysis by HPLC or capillary GLC. The alcohols were then converted to their respective propanoate esters by reaction with propanoic anhydride in pyridine. Table 1

FIG. 5. Synthesis of individual enantiomers of 8-methyl-2-decanol propanoate.

TABLE 1. ISOMERIC COMPOSITION

| | Isomeric composition (%) | | | |
|---|---|---|---|---|
| Isomer | 2*R*,8*R* | 2*S*,8*R* | 2*R*,8*S* | 2*S*,8*S* |
| 2*R*,8*R* | 97.3 | 0.8 | 1.9 | — |
| 2*S*,8*R* | 0.4 | 97.7 | — | 1.9 |
| 2*R*,8*S* | 0.8 | — | 98.3 | 0.9 |
| 2*S*,8*S* | — | 0.8 | 0.4 | 98.8 |

summarizes the configurational composition of the products as determined by analysis of intermediates in the synthetic sequence. The biological activity of these compounds have been reported by Guss et al. (1983b, 1984, 1985).

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# FIELD TRAPPING OF THREE *Epinotia* SPECIES WITH (Z,Z)-7,9-DODECADIENYL ACETATE (LEPIDOPTERA: TORTRICIDAE)

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**Abstract**—Field survey of the geometrical isomers of 7,9-dodecadienyl alcohol, acetate, and aldehyde has resulted in attractants and inhibitors for three species of tortricid moths. *Epinotia silvertoniensis* and an undescribed *Epinotia* sp. were all attracted to (Z,Z)-7,9-dodecadienyl acetate. Another *Epinotia* sp. was attracted to (Z,Z)-7,9-dodecadienyl acetate and (Z,Z)-7,9-dodecadien-1-ol. Electroantennogram data and inhibition patterns for one of the *Epinotia* sp. are also reported. In addition, *E. criddleana* was attracted to lures containing (*E*)-9-dodecenyl acetate.

**Key Words**—(Z,Z)-7,9-Dodecadienyl acetate, (Z,Z)-7,9-dodecadien-1-ol, *Epinotia silvertoniensis*, *Epinotia criddleana*, *Epinotia* sp., Lepidoptera, Tortricidae, sex attractant.

## INTRODUCTION

In our search for new lepidopteran sex attractants, we have field screened general classes of compounds. Surveys of monounsaturated straight-chain primary alcohols, acetates, and aldehydes (monoenes) have uncovered a large number of attractants for Lepidoptera (Steck et al., 1977, 1979, 1982). Our field screening of conjugated diunsaturated straight-chain primary alcohols, acetates, and aldehydes (dienes) has also resulted in chemical sex attractants for a number of moth species. Screening of the four geometrical isomers of 5,7-dodecadienyl alcohol, aldehyde, and acetate produced attractants for three noctuid species (Reed et al., 1984) and one tortricid moth (Chisholm et al., 1985). Screening of the four geometrical isomers of 8,10-dodecadienyl alcohol, acetate, and al-



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dehyde (8,10-dodecadienes) resulted in attractants for 12 Olethreutinae species (Chisholm et al., 1985).

Roelofs et al. (1973) reported isolation of (*E*,*Z*)-7,9-dodecadienyl acetate (*E*7,*Z*9–12:Ac) as a pheremone component of *Lobesia botrana* (D & S) and Chisholm et al. (1985) hâve reported (*E*,*E*)-7,9-dodecadienyl acetate (*E*7,*E*9–12:Ac) + (*Z*,*E*)-7,9-dodecadienyl acetate (*Z*7,*E*9–12:Ac) as a sex attractant for *Eucosma delericta* (Heinr).

We report the synthesis and field survey of all four geometrical isomers of the 7,9-dodecadienyl alcohol, acetate, and aldehyde (7,9-dodecadienes). This survey produced chemical sex attractants for *Epinotia silvertoniensis* (Kft.) and two undescribed *Epinotia* species referred to as *Epinotia* sp. (A) and *Epinotia* sp. (B). *Epinotia criddleana* was attracted to lures containing (*E*)-9-dodecenyl acetate (*E*9–12: Ac) during our replicated experiments.

## METHODS AND MATERIALS

The chemicals used in this study were synthesized and purified in this laboratory. The 7,9-dodecadien-1-ols and the 9,11-tetradecadien-1-ols were synthesized by procedures previously described for the synthesis of the 5,7-dodecadien-1-ols (Chisholm et al., 1981, Palaniswamy et al., 1983). The 9,11-dodecadien-1-ols, dodecen-1-ols, and tetradecen-1-ols were synthesized similarly by Wittig condensation reactions.

The four geometrical isomers of 7,9-dodecadien-1-ol were recovered from Wittig condensation reactions. The *Z*,*E* and *E*,*E* isomers were prepared by condensation of the ylide of 7-acetoxyheptyltriphenylphosphonium bromide with (*E*)-2-pentenal. The *E*,*Z* and *E*,*E* isomers were prepared from the ylide of propyltriphenylphosphonium bromide with (*E*)-9-tetrahydropyranyloxy-2-nonenal. The *Z*,*Z* and *E*,*E* isomers were recovered from the condensation of the ylide of (*Z*)-2-pentenyltriphenylphosphonium bromide with 7-acetoxyheptanal.

The four geometrical isomers of 9,11-tetradecadiene-1-ol were recovered from similar Wittig condensation reactions. The *Z*,*E* and *E*,*E* isomers were prepared by the condensation of the ylide of 9-acetoxynonyltriphenylphosphonium bromide with (*E*)-2-pentenal. The *E*,*Z* and *E*,*E* isomers were prepared from ylide of propyltriphenylphosphonium bromide with (*E*)-11-tetrahydropyranyloxy-2-undecenal. The *Z*, *Z* and *E*, *E* isomers were recovered from the isomer mixture resulting from the condensation of the ylide of (Z)-2-pentenyltriphenylphosphonium bromide with 9-acetoxynonanal.

The *E* and *Z* isomers of 9,11-dodecadien-1-ol were recovered from the Wittig condensation of the ylide of 3-propenyltriphenylphosphonium bromide with 9-acetoxynonanal. The *E* and *Z* isomers of 7-dodecen-1-ol were recovered from the Wittig condensation of the ylide of 7-acetoxyheptyltriphenylphosphonium bromide with pentanal. The *E* and *Z* isomers of 9-dodecen-1-ol were recovered

from the condensation of the ylide of propyltriphenylphosphonium bromide with 9-tetrahydropyranyloxynonanal. (Z)-8-tetradecen-1-ol was recovered from the Wittig condensation of the ylide of 8-acetoxyoctyltriphenylphosphonium bromide with hexanal. After work-up, the geometrical isomers were separated by argentation chromatography (Houx et al., 1974). All aldehydes were prepared by pyridinium chlorochromate oxidation of the corresponding alcohol (Corey and Suggs, 1975). All acetates were prepared by treatment of the corresponding alcohol with 10-fold excess of acetic anhydride at 100°C for 1 hr. The mixture was then cooled and neutralized with water and sodium bicarbonate and extracted with hexane. The compounds were analyzed for structure using 90 MHz NMR, IR spectroscopy, and mass spectrometry. Isomeric purity was checked using capillary gas chromatography (Chisholm et al., 1985), and all compounds used were greater than 98% pure.

Pherocon ICP traps (Zoecon Corp., Palo Alto, California) containing the chemical lures impregnated into rubber septa (Arthur H. Thomas #8753-D22) were hung on tree branches at a height of 1–2 m. Each lure was protected from oxidation by adding two drops of a 10% solution of butylated hydroxytoluene (BHT) in acetone. The traps were inspected and captures recorded twice weekly. Specimens were collected, and sticky liners and lures replaced when necessary.

Data from the randomized three-times replicated field tests were transformed $\sqrt{X+0.5}$ and then were submitted to analysis of variance; significantly different means were separated by Duncan's multiple range test.

Electroantennogram (EAG) response of *Epinotia* sp. (A) to synthetic chemicals was recorded (Chisholm et al., 1975) using field-trapped males recovered from traps within 12 hr of capture.

Field experiments for *E. Silvertoniensis* and *Epinotia* sp. (B) were carried out 100 km northeast of Saskatoon, Saskatchewan, Canada, in a forest area bordering on parkland. The area contains a majority of deciduous trees including white birch, *Betula papyrifera* Marsh., trembling aspen, *Populus tremuloides* Michx., chokecherry, *Prunus virginiana* L., and Saskatoon berry, *Amelanchier alnifolia* (Nutt.); and two species of conifers, white spruce, *Picea glauca* (Moench), and Jack pine, *Pinus banksiana* Lamb. The area also contained rose bushes *Rosa* sp. and a variety of herbaceous shrubs. Trapping for the *Epinotia* sp. (A) was carried out near Saskatoon in a windbreak formed of green ash *Fraxinus pennsylvanica* var. *subintegerrima* (Vahl.).

## RESULTS AND DISCUSSION

A set of nonreplicated survey traps containing single-component and two-component mixtures of the 7,9-dodecadienes, set out in May 1982 and monitored through September, caught 62 *Epinotia* sp. (B) and 450 *E. silvertoniensis*. One hundred nineteen *Epinotia* sp. (A) were caught in single-component survey

traps in April 1983. All three species were attracted to (*Z*,*Z*)-7,9-dodecadienyl acetate (*Z*7,*Z*9–12 : Ac) at 100-μg dose. *Epinotia* sp. (B) was attracted to (Z,Z)-7,9-dodecadien-1-ol (*Z*7,*Z*9–12 : OH) at 100-μg dose. The other geometrical isomers of the alcohol, acetate, and aldehyde were tested as single components at 100-μg dose, and none were attractive. They were also tested as second components with the attractants, and only the isomers discussed had any effect on capture. Tables 1, 2, and 3 show some of the replicated field experiments carried out during the 1983 and 1984 flight periods. The lures in the experiments were selected on the basis of survey or previous experimental results and on a "one-change" (Steck et al., 1982) in chemical structure, differing either in geometry, functional group, or chain length.

The 1982 survey captures indicated that *Z*7,*Z*9–12 : Ac at 100-μg dose was the best lure for *Epinotia* sp. (B); however, replicated data from 1983 showed that *Z*7,*Z*9–12 : OH at 100 μg caught significantly more than *Z*7,*Z*9–12 : Ac (Table 1). In Table 1, the data from the 1984 tests show that the lures where *Z*7,*Z*9–12 : OH was present at 100 μg were not significantly different from *Z*7,*Z*9–12 : Ac as a single component, but were better than any two-component bait with *Z*7,*Z*9–12 : Ac as the major component.

*E. silvertoniensis*, whose flight period overlapped that of *Epinotia* sp. (B) (Figure 1), was also captured by *Z*7,*Z*9–12 : Ac at 100-μg dose. When *Z*7,*Z*9–12 : OH was combined with *Z*7,*Z*9–12 : Ac, capture of *E. Silvertoniensis* was reduced by 90% or more (Tables 1 and 2). If *Z*7,*Z*9–12 : OH is a pheromone

TABLE 1. CAPTURE OF *Epinotia* sp. (B) AND *Epinotia silvertoniensis* BY TRAPS BAITED WITH 7,9-DODECADIENES

| | Total males captured[a] | | |
|---|---|---|---|
| | *Epinotia* sp. (B) | | *E. silvertoniensis* |
| Lure composition (μg) | 1983 | 1984 | 1984 |
| *Z*7,*Z*9–12:OH (100) | 70 e | 92 a | 0 |
| *Z*7,*Z*9–12:Ac (100) | 22 f | 61 ab | 10 |
| *Z*7,*Z*9–12:Ald (100) | 3 f | 4 d | 0 |
| *Z*7,*Z*9–12:OH: (100) + *Z*7,*Z*9–12:Ac (10) | — | 89 a | 1 |
| *Z*7,*Z*9–12:OH (100) + *Z*7,*Z*9–12:Ald (10) | — | 101 a | 0 |
| *Z*7,*Z*9–12:Ac (100) + *Z*7,*Z*9–12:OH (10) | 15 f | 17 cd | 1 |
| *Z*7,*Z*9–12:Ac (100) + *Z*7,*Z*9–12:Ald (10) | 26 f | 36 bc | 4 |
| *Z*7,*Z*9–12:Ald (100) + *Z*7,*Z*9–12:OH (10) | — | 13 cd | 0 |
| *Z*7,*Z*9–12: Ald (100) + *Z*7,*Z*9–12: Ac (10) | — | 18 cd | 3 |

[a]Three times replicated, June 23–July 18, 1983, and June 18–July 16, 1984. Numbers followed by the same letter are not different ($P = 0.05$). (−) indicates that mixture was not tested at that time.

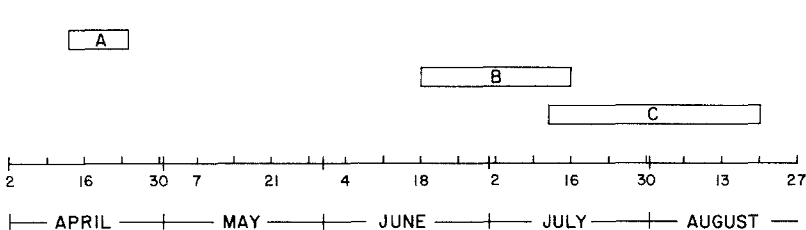


FIG. 1. 1984 Flight periods for *Epinotia sp.* (A), *Epinotia* sp. (B) and *Epinotia silvertoniensis* (C).

component, it could play a role in species isolation of these two closely related species. When (Z,Z)-7,9-dodecadienal (Z7,Z9-12:Ald), the other geometrical isomers of Z7,Z9-12:Ac, and the analogous monoenes (Table 2) were tested as minor components, no significant effect was seen. Twenty-five *Epinotia criddleana* were captured between June 18 and July 4 in replicated traps containing *E*9-12:Ac at 100-μg dose.

*Epinotia* sp. (A) is also attracted to Z7,Z9-12:Ac, but its flight period is separated from the others and so no interference occurs (Figure 1). The single component was found to be as good as any two-component mixture tested. The three geometrical isomers and the analogous monoene acetates were tested as minor components of these, and we found only (*E*,*Z*)-7,9-dodecadienyl acetate (*E*7,Z9-12:Ac) and (Z)-9-dodecenyl acetate (Z9-12:Ac) reduced capture. This resulted in testing of these and other related compounds (Table 3). In all cases tested (Figure 2), capture reduction occurred when the double-bond positioned 3-4 from the terminal carbon (9-10 in C12, 11-12 in C14) was *cis* and the 5-6

TABLE 2. CAPTURE OF *Epinotia silvertoniensis* BY TRAPS BAITED WITH 7,9-DODECADIENES AND VARIOUS MONOENES

| Lure composition (μg) | Total *E. silvertoniensis*[a] males captured |
|---|---|
| Z7,Z9-12:Ac (100) | 121 ab |
| Z7,Z9-12:Ac (100) + Z7-12:Ac (10) | 175 a |
| Z7,Z9-12:Ac (100) + E7-12:Ac (10) | 110 ab |
| Z7,Z9-12:Ac (100) + Z9-12:Ac (10) | 56 bc |
| Z7,Z9-12:Ac (100) + E9-12:Ac (10) | 138 ab |
| Z7,Z9-12:Ac (100) + Z7,Z9-12:OH (10) | 15 cd |
| Z7,Z9-12:Ac (100) + Z7,Z9-12:OH (100) | 2 d |
| Z7,Z9-12:OH (100) | 0 d |
| Z8-14:Ac (100) | 0 d |

[a] Three times replicated, August 3-20, 1984. Numbers followed by the same letter are not different ($P = 0.05$).

TABLE 3. CAPTURE OF *Epinotia* sp. (A) BY TRAPS BAITED WITH 7,9-DODECADIENES AND VARIOUS OTHER DIENES AND MONOENES

| Lure composition (μg) | Total Males Captured[a] | |
|---|---|---|
| | Test 1 | Test 2 |
| *Z*7,*Z*9-12:Ac (100) | 284 a | 93 c |
| *Z*7,*Z*9-12:Ac (100) + *Z*7-12:Ac (10) | 336 a | — |
| *Z*7,*Z*9-12:Ac (100) + *E*7-12:Ac (10) | 313 a | — |
| *Z*7,*Z*9-12:Ac (100) + *Z*9-12:Ac (10) | 29 b | 10 d |
| *Z*7,*Z*9-12:Ac (100) + *E*9-12:Ac (10) | 273 a | — |
| *Z*7,*Z*9-12:Ac (100) + *E*7,*Z*9-12:Ac (10) | 9 b | — |
| *Z*7,*Z*9-12:Ac (100) + Z9,11 – 12:Ac (10) | — | 22 d |
| *Z*7,*Z*9-12:Ac (100) + *E*9,11-12:Ac (10) | — | 69 c |
| *Z*7,*Z*9-12:Ac (100) + *Z*9, *E*11-14:Ac (10) | — | 127 c |
| *Z*7,*Z*9-12:Ac (100) + *Z*9, *Z*11-14:Ac (10) | — | 87 c |
| *Z*7,*Z*9-12:Ac (100) + *E*9, *Z*11-14:Ac (10) | — | 18 d |
| *Z*7,*Z*9-12:Ac (100) + *E*9, *E*11-14:Ac (10) | — | 159 c |

[a] Three times replicated; test 1, April 13-24, 1984; test 2, April 16-24, 1984. Numbers followed by the same letter are not different ($P = 0.05$). (–) indicates that mixture was not tested at that time.

bond from the terminal carbon (7-8 in C12, 9-10 in C14) was not a *cis* double bond. Any other geometry tested had no significant effect on captures including (*E*,*Z*)-7,9-dodecadienyl acetate and (*E*,*E*)-7,9-dodecadienyl acetate. *Epinotia* sp. (A) was also captured in small numbers (40) by (*Z*,*Z*)-8,10-dodecadienyl acetate (*Z*8,*Z*10-12 : Ac) when not in competition with *Z*7,*Z*9-12 : Ac. This attraction may be a substitution because of a similarity of structure.

We can only speculate on whether "specialist" receptors are present for this response or the "inhibiting" compounds are interfering with the key substance. Priesner (1979a,b, 1983) has noted a pattern in responses to single receptor cells that differ between Noctuidae and Tortricidae such that the part of the chain between the apolar alkyl group and the double bond is most critical for a stimulatory effect on receptor cells in noctuids and the other end of the chain is most critical in tortricids. The inhibition of capture here seems to be determined by the structure of the chain including the double bonds and the "nonpolar" end of the molecule.

EAG data (Figures 3 and 4) for *Epinotia* sp. (A) showed strong responses for all the 7,9-dodecadienyl acetates, with *Z*7,*Z*9-12 : Ac giving the strongest response and relatively large responses also recorded for *Z*7,*Z*9-12 : Ald and *Z*7,*Z*9-12 : OH. The *E*9-12 : Ac gave the largest response of all the monoenes tested, but in our field tests (Table 3), when 10 μg *E*9-12 : Ac was added to *Z*7,*Z*9-12 : Ac at 100 μg, there was no significant difference in moth captures.

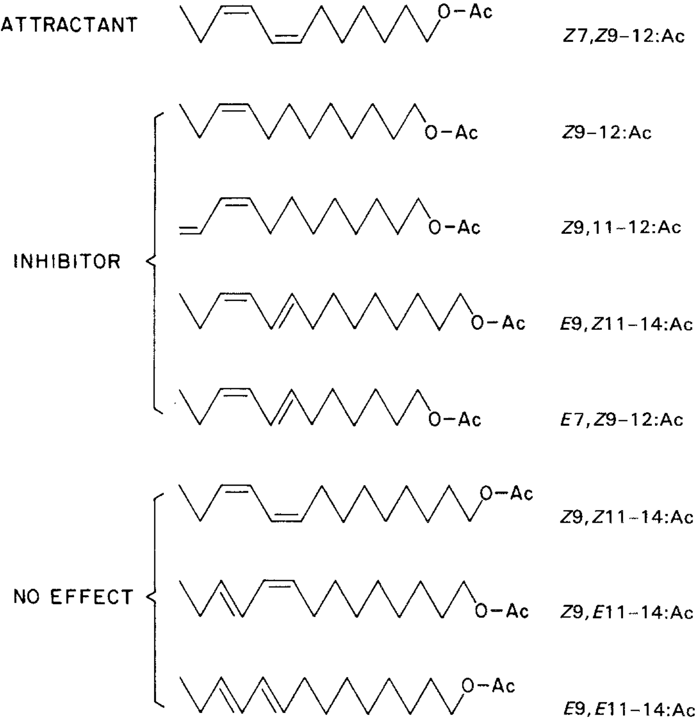


FIG. 2. Structural differences between attractant and inhibitors for *Epinotia* sp. (A).

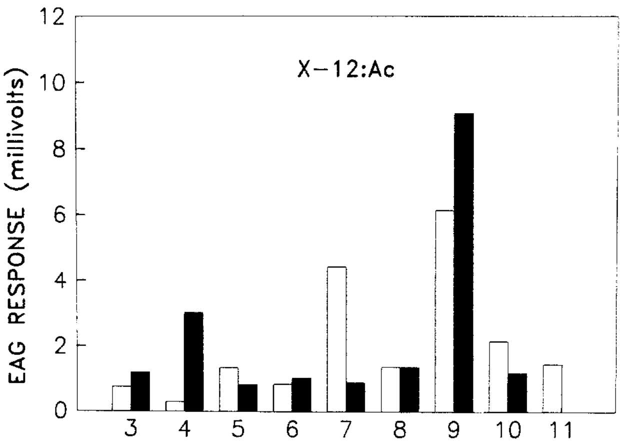


FIG. 3. EAG responses of *Epinotia sp.* (A) males to *Z* (white) and *E* (black) dodecenyl acetates.

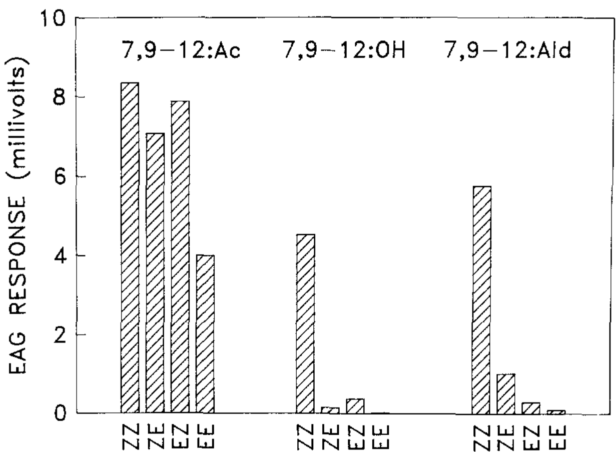


FIG. 4. EAG responses of *Epinotia* sp. (A) males to 7,9-dodecadienyl alcohol, acetate, and aldehyde isomers.

(Z)-7-Dodecenyl acetate (Z7–12 : Ac) and Z7, E9–12 : Ac also gave relatively large responses, but no effect was seen in the field tests. Both Z9–12 : Ac and E7, Z9–12 : Ac gave strong EAG responses, and also reduced moth captures in the field.

*Epinotia* is a large genus of external leaf webbers and leaf rollers, and Hodges et al. (1983) reported 75 described species in North America. Attractants have been reported for five species of *Epinotia*, including the single-component Z7–12 : Ac for *E. atristriga* Clarke and *E. zandana* (Kearfott) (Roelofs and Comeau, 1969), as well as two-component mixtures of (*E*)-8-dodecenyl acetate (*E*8–12 : Ac) + (*E*)-10-dodecenyl acetate (*E*10–12 : Ac) (1 : 1) for *E. nanane* (Treitschke), Z9–12 : Ac + Z7–12 : Ac (1 : 1) for *E. rubiginosana* (Herr.-Schaffer), and E9–12 : Ac + Z9–12 : Ac (9 : 1) for *E. tedella* (Clerk). (Booij and Voerman, 1984).

The similarity of structure of the attractants for the various *Epinotia* sp. is obvious, and it follows the general rule that attractants in the olethreutinae subfamily are 12-carbon compounds. The fact that most of the reported attractants are monoenes may be because dienes have not been as extensively surveyed as the monoenes. In the *Epinotia* genus, all attractants are 12-carbon compounds with unsaturations at the 7 and or 9 position with *E. nanane* being one exception. Chisholm et al. (1985) showed a number of 8,10-dodecadienes as attractants for the olethreutinae species in genera that had been previously reported to be predominantly 8-dodecenyl alcohols and acetates. Further studies involving survey of these and other conjugated dienes may show a more general trend of monoene–diene interaction in the Tortricidae family and others.

The action of Z9–12 : Ac interfering with attraction of *Epinotia* sp. (A) is clear, and there is a growing number of examples of monoene–diene combina-

tions as sex attractants or pheromones in Lepidoptera. For example, a combination of *Z*7–12 : Ac and (*E,Z*)-5,7-dodecadienyl acetate at a ratio of 1 : 1 is necessary to attract *Oncocnemis cibalis* (Reed et al., 1984). Roelofs and Weires (Roelofs and Brown, 1982) have reported *Hedya chionosema* (Zeller) attracted to (*E,E*)-8,10-dodecadienyl acetate (*E*8,*E*10–12 : Ac), *Z*8–12 : Ac, and *E*8–12 : Ac at a ratio of 100 : 35 : 3. Frérat et al. (1979) have isolated pheromone components of *Hedya nubiferana* (Haworth) and identified them as *E*8,*E*10–12 : Ac, *Z*8–12 : Ac, *E*8–12 : Ac, and dodecyl acetate in a ratio of 55 : 32 : 5 : 9. *Rhyacionia frustrana* (Comstock) has a pheromone with (*E*)-9,11-dodecadienyl acetate and *E*9–12 : Ac as two components at a ratio of 4 : 96 (Hill et al., 1981). Dienes may also be important in the isolation of species that have been difficult to catch selectively with monoenes.

The discovery and reporting of attractants for undescribed species will be a useful aid to the taxonomist by providing an easy method of collecting specimens and an aid to biologists in general in discovering its habitat and studying its behavior.

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# MULTICHEMICAL DEFENSE OF PLANT BUG *Hotea gambiae* (Westwood) (HETEROPTERA: SCUTELLERIDAE): (*E*)-2-Hexenol from Abdominal Gland in Adults

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**Abstract**—The occurrence in *Hotea gambia* adults of a sexual dimorphism in the divided dorsal abdominal scent gland (dg 1)is reported. Counts made of ducted secretory units indicate that female dg 1 regresses at the end of larval development, unlike male dg 1 which undergoes no regression. Other dorsal abdominal scent glands (dg 2, dg 3) which function in the larvae cease to function during the imaginal moult. From gas chromatographic, mass spectrometric and [$^1$H]NMR data, the identity of the secretion from male adult dg 1 was established as virtually pure (*E*)-2-hexenol (a 100-mg mature male adult contains 0.5–1 μl of secretion). 2-Hexenol was also found in the reduced female adult dg 1. In the sexually monomorphic metathoracic scent gland, (*E*)-2-alkenals ($C_6$, $C_8$) and (*E*)-4-oxohex-2-enal, together with monoterpenes (β-pinene, limonene) but not 2-hexenol, were identified. The vapor of (*E*)-2-hexenol is repellent to both sexes of *Hotea* adults and toxic to blowfly (*Calliphora*) eggs.

**Key Words**—Scent gland, defensive secretion, *Hotea gambiae*, Heteroptera, Scutelleridae, (*E*)-2-hexenol, hex-2-enal, oct-2-enal, 4-oxohex-2-enal, β-pinene, limonene, sex dimorphism, feeding, repellent, pheromone, ovicide, *Calliphora*, Diptera, Calliphoridae.

## INTRODUCTION

A sexual dimorphism in those scent glands (dorsal abdominal, metathoracic) which, in many species of Heteroptera are known to have an ecological role in defense against predation (Remold, 1962), although unusual, has been recorded



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in the adult in representatives from four different families (Belostomatidae-Lethocerinae, Lygaeidae, Enicocephalidae, in the metathoracic scent gland; Pentatomidae-Asopinae, in the dorsal abdominal scent gland) (Aldrich et al., 1978, 1984; Carayon, 1948, 1971; Dupuis, 1952, 1959; Games & Staddon, 1973; Johannson, 1957; Staddon, 1971, 1979). The hypermorphic gland is always present in the male adult. Although various suggestions have been devised to explain the biological significance of sex dimorphisms in scent glands, none, so far as we are aware, has been rigorously confirmed as yet.

In *Hotea gambiae*, the metathoracic scent gland is sexually monomorphic. However, the divided dorsal abdominal scent gland, which corresponds to dg 1 in the larvae (Gough et al., 1985), shows a pronounced sexual dimorphism in size. In this paper we report on the chemical composition of the secretions from the two adult scent glands. The chemistry of the larval system of glands has been the subject of a previous paper (Gough et al., 1985). Developmental observations on all three abdominal scent glands in *H. gambiae* are also reported. There was interest in deciding whether selective changes in gland size were occurring during development in dg 1 in the female, in the male, or in both sexes. Finally, we describe experimental findings which supply possible clues to the role of dg 1 in the ecology of *H. gambiae.*

## METHODS AND MATERIALS

Methods of insect rearing and chemical microanalysis have been described in a previous paper (Gough et al., 1985).

*Ductule Counts.* Individual glands for ductule counts were prepared by removal of all soft tissues in caustic potash. Surviving cuticular material (cuticular intima, cuticular ductules of the multicellular secretory units) was stained lightly with Azo black and mounted in Euparol for examination by ordinary light microscopy. Individual ductules are ca. 7 $\mu$M long, 1 $\mu$M in diameter, and show a conspicuous swelling (ca. 5 $\mu$M in diameter) apically. Preparations were sufficiently transparent to enable ductules to be seen and counted on the lower, as well as the upper, surface of the preparation. Ductule counts were made for all three glands in all five larval instars and for the separate sexes in fifth-stage larvae which can be sexed from differences in the abdominal venter. The growth ratio ($r$) for adjacent instars was estimated from sample means ($N$, at least 4). For example, the ratio found from sample means for male dg 1 instar 5 to male dg 1 instar 4 was 742/389. Hence $r = 1.9$ in this case. The coefficients of variation ($CV$) of the sample means varied about a mean $CV$ of 22%.

*Repellency Tests.* The vapor stimulus [($E$)-2-hexenol; Aldrich] was applied, in the vicinity of the head, to a resting adult (exposure time 30 sec) from the tip of a glass capillary (ID 1 mm). The occurrence or not of an avoidance reaction (movement away from the stimulus source) was recorded. A movement towards

the stimulus source indicating attraction was never observed. Tests were carried out on 40 individual adults (test group, $N = 20$; control group, $N = 20$). One test was carried out on fasted, the other carried out on fed adults. A clean capillary supplied the stimulus blank for adults in the control group. Applications of test and stimulus blank were randomized. The null hypothesis that the proportions in the test and the control groups were the same was tested using the $\chi^2$ distribution.

*Feeding Tests.* The responsiveness of bugs to a source of pure (*E*)-2-hexenol vapor in comparison with an olfactory stimulant (geranyl acetate) and a comparable odor-free source was investigated using the fact that feeding through a surface involves prior deposition of a visible stylet sheath (Miles, 1972). The experimental system used four open breeding containers (22 × 32 cm floor dimensions and 20 cm high) each containing between 20 and 100 adults and larvae in free movement on the paper debris lining the floor of the container. Three groups of three glass microflasks (total capacity of individual flasks ca. 50 μl) were prepared for each breeding container: (1) a test group containing 5 μl of (*E*)-2-hexenol (Aldrich) per flask; (2) a feeding control group containing 5 μl of a geranyl acetate mixture per flask (citronellyl acetate was also present in this sample of geranyl acetate); and (3) a blank control group consisting of three empty flasks. To minimize possible position effects, the flasks were secured on the floor of the breeding containers in a 3 × 3 Latin square design (distance between flasks in columns and rows, 5 cm). The long neck of the flask raised the opening (ID ca. 1 mm) 29 mm above the level of the cardboard flask-support system.

After 48 or 72 h in the breeding container (temperature 26°C), the numbers of stylet sheaths on the necks of flasks in all three groups were counted. Stylet sheaths are individually 0.1 mm long and 0.05 mm across and easily counted. We wanted to compare stylet sheath deposition in the feeding control group and the hexenol and blank control groups. Greater deposition of stylet sheaths on the feeding control would indicate the occurrence of feeding activity and olfactory discrimination in the event that stylet sheath deposition on hexenol test and blank control groups was low. Secondly, we wanted to compare stylet sheath deposition in the hexenol test and blank control groups. To make these comparisons, a model I three-way ANOVA with replication ($r = 4$) was used. Data were converted to percentages and transformed using an arcsine transformation (Sokal and Rohlf, 1981; Box et al., 1978).

*Calliphora Egg Toxicity Tests.* Freshly laid eggs of blowfly *Calliphora erythrocephala* on the ox liver on which they had been laid were divided into small groups. The mean egg batch size was 39 and the range 20–66, but the counts were not made until the end of the experiment in order to minimize mortality from handling. Stoppered glass sample tubes (capacity 18 ml) were divided into test and control groups. An atmosphere containing a measured 1.6 mg hexenol/liter of air was obtained by placing filter paper strips soaked with

(*E*)-2-hexenol (Aldrich) in the sample tubes in the test groups. Unwetted strips were placed in the sample tubes in each control group. In the test groups, care was taken to prevent the occurrence of possible contacts between the egg batch and liquid hexenol. To examine the change in mortality with time during exposure to hexenol vapor, exposure times of 3, 6, and 12 hr were selected. Preliminary tests indicated that an exposure time of 24 hr was highly deleterious to the eggs. To determine percentage mortalities, batches of eggs were transferred to clean tubes and the numbers of unhatched and hatched eggs determined after two days. Normally, hatching occurs within 24 hr of laying at 26°C. Probit analysis (Finney, 1964) of percent mortality was undertaken to estimate the $LD_{50}$ in hours of exposure to (*E*)-2-hexenol vapor.

## RESULTS

### *Morphology*

The divided dorsal abdominal scent gland (Fig. 1A) in the adult bug derives ontogenetically from dg 1 (Gough et al., 1985) in the larva. Like the larval dg 1, adult dg 1 appears to be pigmentless, and the ducts of the multicellular secretory units open directly through the cuticular gland lining into the central reservoir. The external opening of dg 1 in the adult is concealed from view by the scutellum which is extensive. Hence, the manner of emission of secretion from adult dg 1 could not be examined. The female adult dg 1 is much smaller than the male and, in older adults, often flattened as though containing little or

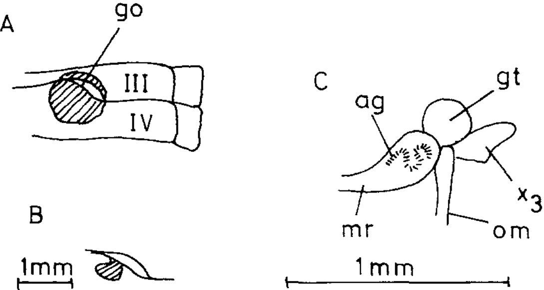


FIG. 1. Adult scent glands of *H.* gambiae, right-hand side. (A) Male dg 1 (dorsal abdominal scent gland), shaded area. (B) Female dg 1, shaded. (C) Metathoracic scent gland, right-hand side (left- and right-hand sides of the median reservoir are joined in the midline). ag, accessory gland; go, gland orifice; gt, pigmentless mass composed of gland secretory tubules; mr, median scent reservoir, pigmented; om, dorsoventral opener muscle; and $x_3$, metasternal apophysis. Roman numerals, tergites of third and fourth abdominal segments.

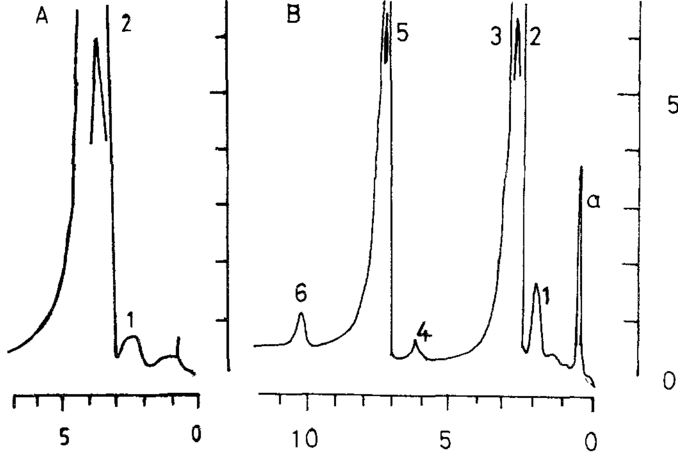


FIG. 2. Gas chromatograms of secretion from (A) male adult dg 1 and (B) metathoracic scent gland. Open column injection. Horizontal scale, time from injection in minutes. Vertical scale, detector response in arbitrary units. Range and attenuation: (A) and (B) $8 \times 10^{12}$. Oven, 70°C for 5 min and then temperature programmed at 6°/min to 200°C. Column, 2 m × 2 mm ID glass packed with 3% OV-225 on 60–80 mesh Gas Chrom Q. a, air peak.

no secretion (Figure 1B). Peak area response (GC) comparisons indicate that female dg 1 contains 1000 times less material than the male dg 1.

The metathoracic scent gland (Figure 1C) is of a type peculiar to Pentatomoidea in showing, in addition to the usual bundles of secretory tubules and median scent reservoir, a markedly lineariform accessory gland in the wall of the median scent reservoir (see Brindley, 1930; Henrici, 1940; Remold, 1962; Carayon, 1971, among others for descriptions of this organ among Pentatomoidea). The secretory tubules appear to be pigmentless; the epithelium of the median reservoir is pigmented a lemon-yellow color. A biosynthetic role for secretion from the accessory gland in the formation of scent gland aldehydes in the metathoracic scent gland has been indicated (Gilby and Waterhouse, 1967; Games and Staddon, 1973; Aldrich et al., 1978).

## *Chemical Findings*

Gas chromatograms of secretion from an adult male (dg 1 and metathoracic scent gland) are shown for comparison in Figure 2. Dg 1 (Figure 2A) showed a major peak (peak 2, 99.9% of total) and one quite minor peak (peak 1). The metathoracic scent gland (Figure 2B) showed five peaks. The second peak (peak 2,3) was shown by EI-GC-MS to be a mixture of two components.

*Male Adult dg 1.* The EI mass spectrum of the major peak (Figure 2A, peak 2) showed a base peak at $m/z$ 57, significant ions at $m/z$ (intensity, %) 41

(46), 43 (19), 82 (18), 67 (16), 55 (12), 56 (11), 71 (10), 72 (4), and $M^+$100 (2), matching that of authentic (*E*)-2-hexenol (Aldrich). The findings from EI-GC-MS were supported by GC retention tests (OV-225) and [$^1$H]NMR. The NMR spectrum showed signals at $\delta$ 5.65 (1H, dt, *J*-15.5, 5.1 Hz) and $\delta$ 5.69 (1H, dt, *J*-15.5, 5.8 Hz) indicating the *E*-configuration for the double bond at C-2. No analytical data could be obtained for minor peak 1. Evidently, male adult dg 1 contains virtually pure (*E*)-2-hexenol.

*Female Adult dg 1.* Analysis of dg 1 from two female adults by capillary GC (OV-101) yielded two very small peaks. The presence of 2-hexenol was subsequently confirmed by EI-GC-MS of material extracted (solvent, acetone) from five female glands.

*Metathoracic Scent Gland.* The several compounds identified from GC and mass spectral data (Table I) were familiar from analyses made previously on larval dorsal scent glands (Gough et al., 1985). Limonene (Figure 2B, peak 2) and (*E*)-2-hexenal (Figure 2B, peak 3) were unresolved in the original RIC. They were resolved by computer enhancement (Biller and Biemann, 1974). Selected ion monitoring at *m/z* 136 indicated the presence of limonene in amounts

TABLE 1. EI-GC-MS DATA, METATHORACIC SCENT GLAND (mg), MALE ADULT

| Peak (min)[a] | Identity | Peak area (%) | Mass spectrum[b] *m/z* (% abundance) |
|---|---|---|---|
| 1 (2:03) | $\beta$-Pinene[c] | 3 | 93(100), 41(51), 77(26), 79(20), 91(16), 94(12), 80(10), 121(9), 67(9), 107(4), 136(M+, 4) |
| 2 (3:04) | Limonene[c] | 39 (peaks 2 and 3) | 68(100), 67(46), 93(42), 79(20), 53(19), 94(17), 41(15), 121(13), 107(12), 77(11), 91(10), 136(M+, 9), 92(9) |
| 3 (3:27) | (*E*)-2-Hexenal[c] | | 41(100), 55(84), 69(62), 57(53), 83(42), 43(26), 70(22), 56(21), 98(M+,19), 97(11), 80(9) |
| 4 (8:30) | (*E*)-Oct-2-enal[c] | 1 | 41(100), 55(94), 70(79), 57(52), 83(47), 42(41), 69(33), 82(30), 97(13), 93(8), 98(6) |
| 5 (9.54) | (*E*)-4-Oxohex-2-enal | 38 | 83(100), 55(54), 84(19), 57(18), 112(M+, 10), 56(10), 54(6), 53(5), 69(3), 97(2) |
| 6 (16.53) | (*E*)-2-Decenal[c,d] | 19 | 43(100), 55(82), 70(78), 57(62), 83(57), 69(42), 56(35), 98(22), 81(21), 68(20), 67(20), 97(17), 110(12), 121(3), 136(2) |

[a]Retention time; OV-225, 70°C (8 min), then temperature programmed at 6°C/min.
[b]70 eV; above *m/z* 40.
[c]A molecular weight of 154 was indicated by Ci-GC-MS.
[d]Confirmed by comparison (GC, EI-GC-MS) with authentic standard.

similar to β-pinene. No difference (GC, EI-GC-MS) in male and female metathoracic scent gland samples could be found. (*E*)-2-Hexenal is a major component of this secretion.

Estimates of total peak area responses (GC) for all three dorsal glands were obtained. A doubling or a trebling of the volume of stored secretion from one larval instar to the next was indicated by these data. However, the increase in volume in male dg 1 from the fifth larval instar to the adult was much greater (an approximately 15-fold increase was observed. The cuticular intima perhaps undergoes greater unfolding or is more extensive in male adult dg 1 than in the larva.

## *Ductule Counts*

Estimates of number of cuticular ductules (log sample means) on instar number (larval instars 1–5 and the adult) are shown for all three dorsal abdominal scent glands (dg 1, dg 2, and dg 3) in Figure 3. Growth ratios for ductule increase in adjacent instars are also shown where appropriate on the graphs. For normal growth, $r = 2.5$ is indicated by these results. A delay in growth in male dg 1 (Figure 3A) is indicated by $r = 1.1$ for dg 1 instar 2/dg 1 instar 1. Thereafter, growth in the male dg 1 proceeds apparently normally through larval development into the adult. In the female, in contrast, dg 1 regresses late in larval development, and there are relatively few secretory ductules in the adult gland. Evidently, the sex dimorphism in *Hotea* dg 1 is a result of selective atro-

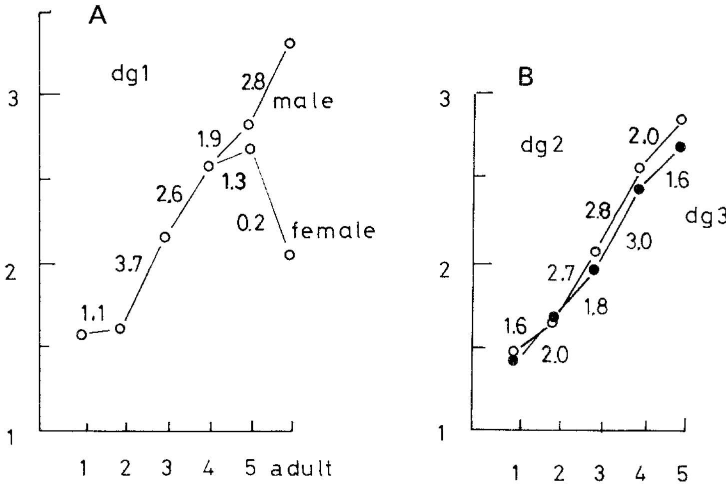


FIG. 3. Log mean number of ductules on instar number for all three abdominal scent glands (dg 1, dg 2, dg 3). The growth ratios marked on the graphs were calculated from the means. The means for dg 1 were calculated from total ductules for dg 1 left and dg 1 right.

phication in the female rather than by hypertrophication in the male. Glands dg 2 and dg 3 (Figure 3B) show similar slightly S-shaped patterns of increase in ductule number but fail to acquire new multicellular secretory units during the imaginal moult and so entirely cease to function in the adults.

### *Behavioral Observations*

A repellent action of (*E*)-2-hexenol vapor was observed on fed and fasted *H. gambiae* adults in the repellency tests using a point source of the vapor ($\chi^2$ analysis, $p < 0.05$). A toxic action was observed when *Hotea* adults were exposed to the vapor of (*E*)-2-hexenol in stoppered tubes. Symptoms of intoxication included proboscis extension, copious release of saliva, and grooming reactions. In a few cases a "knock-down" action of the vapor was recorded. Similar effects on behavior were found to occur within one minute after application of a 1-$\mu$l droplet of (*E*)-2-hexenol into the subscutellar space. In the stylet sheath deposition experiments a selective action of (*E*)-2-hexenol vapor on stylet sheath deposition could not be detected. A significantly greater deposition of stylet sheaths occurred on the flasks containing the geranyl acetate feeding control mixture (Table 2).

### *Toxicity of* (E)*-2-Hexenol Vapor to Blowfly Eggs*

Percentage mortality of blowfly eggs in air saturated with (*E*)-2-hexenol vapor at 26°C at 3, 6, and 12 hr is shown in Figure 4. From the equation of the probit line calculated by the iterative weighted regression procedure (Finney, 1964), the $LD_{50}$ for exposure time to (*E*)-2-hexenol vapor was found to be 3.16 hr. A search for possible delayed deleterious effects of hexenol poisoning by following growth in surviving larvae was not attempted.

TABLE 2. MODEL 1 THREE-WAY ANOVA WITH REPLICATION APPLIED TO STYLET SHEATHS DEPOSITED ON BLANK CONTROL (A), HEXENOL TEST (B), AND GERANYL ACETATE FEEDING CONTROL (C).

| Source of variation | *df* | MS |
|---|---|---|
| Treatments | 2 | 1140.00[a] |
| A + B vs C | 1 | 2272.50[a] |
| A vs B | 1 | 7.59NS[b] |
| Within groups | 20 | 43.95 |

[a] $P < 0.01$.
[b] NS, not significant.

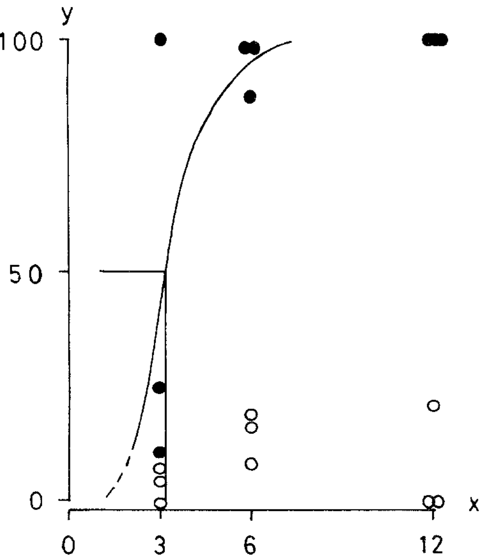


FIG. 4. Percentage mortality in blowfly eggs (*Calliphora erythrocephala*) exposed to a saturated atmosphere of (*E*)-2-hexenol vapor for 3, 6, and 12 hr. Closed circles, hexenol test data. Open circles, control data (hexenol absent). The quantal response curve was fitted to the test data (closed circles) by probit analysis of the 3-hr, 6-hr, and 12-hr sample means. *y* axis, percentage morality; *x* axis, exposure time in hours at 26°C.

## DISCUSSION

The biological significance of (*E*)-2-hexenol production by dg 1 in *Hotea* adults remains to be elucidated. A defensive role could be indicated by the repellency of the vapor as observed in the repellency tests using *Hotea* adults. The observed toxicity of (*E*)-2-hexenol vapor to blowfly eggs indicates a possible role for the alcohol as an ovicidal defense against phasiine parasitoids. Instances of oviposition by phasiines in the subelytral space in their heteropteran hosts have been reported (Dupuis, 1963). However, we have not so far been able to find any records of occurrence of phasiine eggs on body surfaces in *Hotea* adults or to investigate the defensive behavior of *Hotea* adults in nature.

In nature (*E*)-2-hexenol occurs widely as a component of the "green odor" emitted by flowering plants (Visser, et al., 1979). Orientation and feeding reactions mediated by (*E*)-2-hexenol and other green-odor components presumably occur widely among insect herbivores. If any selective action of hexenol vapor occurs none was detected in our stylet sheath deposition experiments using *Hotea* adults.

The occurrence of a sexual dimorphism for (*E*)-2-hexenol production in *Hotea* adults also awaits an explanation. A pheromonal role for (*E*)-2-hexenol in the sexual activities of the adults is possible but has not yet been looked for. As a male-produced repellent, the role of (*E*)-2-hexenol could be to reduce interference among mating pairs by unmated males searching for a female. Perhaps male *Hotea* adults, because of sex differences in apparency or longevity, are in greater need of chemical defense against predators than female adults. Male-biased parasitism by phasiine parasitoids on heteropteran insects has been reported (Mitchel and Mau, 1971; Aldrich et al., 1984). Or, more simply, perhaps the reduction in the female is to be correlated with her greater metabolic needs for reproduction.

We have been informed that a sex dimorphism in adult dg 1 occurs sporadically among scutellerid bugs (J. Carayon, personal communication). Elsewhere among Pentatomoidea, it has been recorded in species of Pentatomidae-Asopinae (Dupuis, 1962, 1959; Aldrich et al., 1978, 1984). In *Podisus maculiventris*, (*E*)-2-hexenal and $\alpha$-terpinol occur as major components in the hypermorphic dg 1 of adult males, and (*E*)-2-hexenol has been detected in this gland (Aldrich et al., 1978). The occurrence of biochemical sex differences in dg 1 in *Podisus* adults has also been reported (Aldrich et al., 1984). In the pentatomine pentatomid *Nezara viridula*, where no sex dimorphism occurs, adult dg 1 produces (*E*)-2-hexenal and *n*-tridecane (Aldrich et al., 1978).

The secretion from the sexually monomorphic metathoracic scent gland of *H. gambiae* is undoubtedly capable of exerting a protective action against small arthropod predators by virtue of the repellency or toxicity of its volatile constituents (Blum, 1981). Interestingly, our failure to detect alkanes in the metathoracic scent gland in *H. gambiae* and a reduction in the size of the median reservoir could be correlated (cf. *Nezara viridula*; Gilby and Waterhouse, 1965). Unicellular secretory units in the wall of the median reservoir could be the source of secreted alkanes in *Nezara viridula* (Gilby and Waterhouse, 1967; Staddon, 1979), and these units are perhaps absent from the median reservoir in *Hotea*.

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# INHIBITION OF SEEDLING GROWTH OF CROP SPECIES BY RECIRCULATING ROOT EXUDATES OF *Bidens pilosa* L.[1]

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**Abstract**—The allelopathic effects of root exudates of *Bidens pilosa* L. on seedling growth of *Lactuca sativa* L., *Phaseolus vulgaris* L., *Zea mays* L., and *Sorghum bicolor* (L.) Moench were studied using a root exudate recirculating system that allows continuous exposure of crop plants to allelopathic chemicals. This system maintains an undisturbed rhizosphere and eliminates competition and physical contact between the donor and acceptor plants. Comparison of responses to hydrophobic and hydrophilic root exudates is made possible by removal of hydrophobic compounds using XAD-4. Treatments consisted of *B. pilosa, B. pilosa* with an Amberlite XAD-4 resin column attached to the donor pot to remove hydrophobic allelochemicals, and a donor pot without weeds. *B. pilosa* significantly inhibited seedling growth of all crop species tested. The crop species varied in response to the root exudates, with *L. sativa* being most sensitive. Larger and older *B. pilosa* plants caused greater inhibition of seedling growth of *L. sativa* and *P. vulgaris* than did smaller (younger) *B. pilosa* plants. *B. pilosa* with XAD-4 caused significantly less inhibition to all crop species, except *Z. mays,* than *B. pilosa* without XAD-4, indicating that the hydrophobic exudates played an important role in the allelopathic growth inhibition. Variability in species response to *B. pilosa* with and without XAD-4 was probably due to differences in sensitivity to hydophobic and hydrophilic allelochemicals.

**Key Words**—Allelopathy, weeds, *Bidens pilosa* L., *Lactuca sativa* L., *Phaseolus vulgaris* L., *Zea mays* L., *Sorghum bicolor* (L.) Moench, root exudates, soil organics, rhizosphere.

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## INTRODUCTION

The Compositae form a large family representing many ecosystems. Some of the Compositae are serious weeds that compete successfully with agricultural crops (Holm et al., 1977). A number of species have demonstrated allelopathic potential and produce compounds with phytotoxic activity (Heywood et al., 1977). *B. pilosa* is a common Compositae weed throughout the humid tropics and is a principal weed of many agricultural crops (Holm et al., 1977).

Among the naturally occurring substances from the Compositae are polyacetylenes and their thiopene derivatives (Bohlmann et al., 1973). *B. pilosa* has been shown to produce some of these compounds (Wat et al., 1979; Bohlmann et al., 1973). Polyacetylenes and their aromatic thiopene derivatives varied between taxa but not within taxa of species of Hawaiian *Bidens,* and the complement of polyacetylenes in roots and leaves was found to be a valuable taxonomic tool (Marchant et al., 1984). Polyacetylenes were not found in the leaves of 13 taxa, but were found in the roots of all. No qualitative variation in polyacetylene production was observed with changes in season or reproductive state of the plants (Marchant et al., 1984). Phenylheptatriyne extracted from leaves of *B. pilosa* inhibited seedling growth of selected test weed species, and the allelopathic activity was enhanced in the presence of sunlight or sources of near-UV light (Campbell et al., 1982). Many other secondary metabolites with possible allelopathic activity have also been isolated from the Compositae (Heywood et al., 1977).

A variety of methods have been used to study the allelopathic potential of roots including aqueous leacheates of whole roots (Chou and Walker, 1980; Bonner and Galston, 1944), aqueous extracts of macerated roots (Wilson and Rice, 1968; Lehle and Putman, 1982), and solvent extracts of macerated roots (Muir and Majak, 1983). Soil has been collected from under plants suspected of producing allelopathic root exudates and placed in pots to test inhibition of seedling growth (Alsaadawi and Rice, 1982; Lolas and Coble, 1982). Solvent extraction procedures have been used to obtain allelochemicals from soil (Carballeira and Cuervo, 1980). Plants have been established in pots with donor and acceptor species separated by dividers to allow mutual exchange of root exudates between donor and acceptor plants (Gilliland and Hayes, 1982). Another system consisted of a U tube containing aerated nutrient solution, with donor and acceptor plants supported at opposite ends (Alsaadawi and Rice, 1982). Dish trays containing nutrient solution were used to culture donor plants, and the nutrient solution was later transferred to jars to test seedling growth (Irons and Burnside, 1982).

A method of passing nutrient solution between pots containing donor plants and pots containing test species was developed by Börner (1958). More recently, this technique has been modified into a staircase system with pots containing donor plants and acceptor test species placed alternately in series. Nutrient so-

lution was allowed to flow through the series of pots for 4–5 hr each day and drain into a tank at the bottom of the staircase. The solution was either added again to the uppermost pot manually (Dunevitz and Ewel, 1981) or pumped to a storage reservoir and later released (Wilson and Rice, 1968; Bell and Koeppe, 1972). Hydrophobic inhibitors were collected for bioassay and chemical identification from the undisturbed root system of *Hemarthria altissima* using XAD-4 resin (Tang and Young, 1982).

Grinding roots for extraction of endogenous substances may liberate chemicals that would not ordinarily escape into the environment. Removal of root systems from soil would damage fine roots and release chemicals that normally remain within the root tissues. Chemical extraction procedures may create artifacts or alter compounds of interest, particularly if these compounds are relatively unstable. Soil collected from under plants may contain substances produced by organisms other than the allelopathic plants of interest, including microorganisms. Collecting soil from under plants could also break roots. Culturing plants hydroponically with aeration may alter the root physiology and cause damage through motion caused by air bubbles, while hydroponic culture without aeration would subject the roots to suboxidation. Under natural conditions, roots continuously release exudates, and the roots of neighboring plants are constantly exposed to these compounds. The term root exudates is used throughout this report because, although the recirculating nutrient solutions undoubtedly contained organic compounds produced by rhizospheric microorganisms and decomposing root residues, the major source of substances is assumed to have been the root exudates of the *B. pilosa* plants.

To study allelopathy, it is important to isolate allelopathy of plants of interest from other factors, and within this limit simulate natural conditions as much as possible. Our objectives were to show, while using undisturbed conditions, the effects of allelopathic root exudates of *B. pilosa* on early plant growth of selected crop species and the effects of removing the hydrophobic exudates. It is also valuable to show the effects of adding back the trapped hydrophobic allelochemicals and ultimately to chemically identify them.

## METHOD AND MATERIALS

*Root Exudate Recirculating System.* Donor pots containing weeds and acceptor pots containing crop plants were made from 1-gallon brown glass solvent bottles with bottoms removed. Acceptor pots were cut 10 cm shorter than donor pots (Figure 1).

Both donor and acceptor pots contained coarse grade silica sand as a rooting medium, with an 8-cm layer of crushed basaltic rock (2 cm size) under the sand. A thin layer of glass wool and a perforated Teflon disk were placed below the crushed rock. The silica sand in acceptor pots was topped with 1-cm No. 2 grade vermiculite to maintain even moisture to germinating seeds.

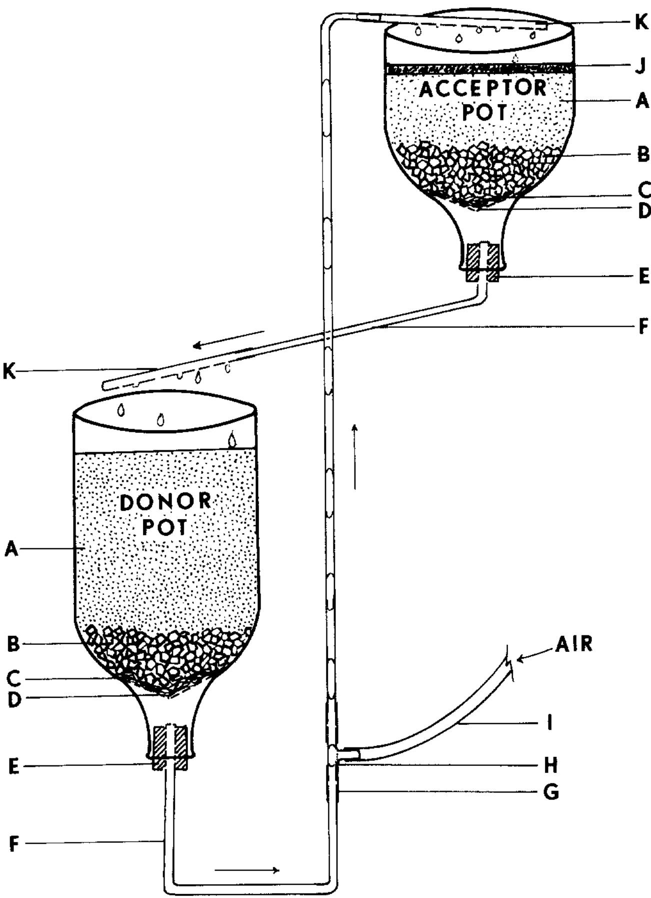


FIG. 1. The root exudate recirculating system. A = silica sand, B = crushed basaltic rock, C = glass wool, D = perforated Teflon disk, E = rubber stopper wrapped with Teflon sealant tape, F = glass tubing, G = Teflon sleeve connector, H = glass T, I = Teflon tube to air pump, J = vermiculite, K = perforated Teflon tube. Arrows indicate direction of flow. A glass column containing XAD-4 resin was attached to the bottom of the donor pot for resin controls.

One-half strength Hoagland's No. 2 nutrient solution (Hoagland and Arnon, 1950) was recirculated through both containers. The acceptor pot was elevated relative to the donor to allow return of nutrient solution to the donor pot by gravity and to prevent shading of acceptor seedlings by donor weeds. Nutrient solution from the donor pot was air-lifted through 6-mm-diameter glass tubing to the acceptor pot using an air pump. Glass tubing was connected to the bottom of containers with bored rubber stoppers wrapped with Teflon sealant

tape. Teflon tubing was used for air inlet lines and glass tubing connections. Containers and glass tubings were wrapped with aluminum foil to exclude light. The solution was circulated at a rate of about 1 liter/hr.

In the resin control pots, hydrophobic allelochemical compounds were removed from the recirculating solution using an XAD-4 column adapted from Tang and Young (1982). The nutrient solution passed through an 18 × 150-mm glass column packed with 12 g Amberlite XAD-4 polymeric adsorbent resin (Rohm and Haas) prior to being air-lifted to the acceptor pot. Since the XAD-4 resin has a high surface area and adsorbent capacity for hydrophobic organic substances in aqueous systems, and many allelopathic compounds have been shown to be hydrophobic secondary metabolites (Whittaker and Feeny, 1971), the use of XAD-4 allows the comparison of effects of donor on acceptor plants with and without the hydrophobic allelopathic component.

*Expermental Design.* Treatments consisted of *B. pilosa* as the donor species, *B. pilosa* with a column containing XAD-4 (resin control), and a donor pot with culture media but without the weeds (pot control). Ten *B. pilosa* plants, from a uniform 60-day-old stand, were established in donor pots. The weeds were cut back to 20 cm above donor pots once established and again one week prior to each run of the experiment. Resin control columns were changed every other day.

Acceptor species, *L. sativa, P. vulgaris, S. bicolor,* and *Z. mays,* were chosen because they represent a wide range of important crops. Acceptor seeds were placed below vermiculite in acceptor pots, one species per pot, and thinned to eight uniform seedlings after emergence.

The salinity (Solu Bridge model RD-B15 salinity meter, Beckman) and pH of the recirculating solutions were monitored every other day. Nutrient levels were maintained at 0.5-strength Hoagland, and pH at 7.0 with $H_2SO_4$. Recirculating systems were started three days prior to seeding to allow accumulation of allelochemics, and solutions were not changed during the experiment except for replenishing water and Hoagland's solution.

One recirculating system (donor + acceptor) was used for each acceptor species × treatment combination. The experiment was run twice (replicated with time). Replication 1 began when *B. pilosa* plants were approximately 81 days old and 21 days after transplanting to donor pots. Replication 2 was started 28 days after replication 1 was started. The same pots of *B. pilosa* plants were used for both replications and were randomly assigned to different acceptor pots for each replication. Data were collected daily beginning the fifth day after seeding (first day after emergence) on plant height for *Z. mays* and *S. bicolor,* and leaf area for *P. vulgaris* and *L. sativa.* For all acceptor species except *L. sativa,* data were collected for 10 days or through the 14th day after seeding, and treatments were compared statistically, independently for each acceptor species at each date. Data were analyzed as randomized block design analyses with time

periods (runs) as replications. *L. sativa* was terminated 11 days after seeding for replication 1 and seven days after seeding for replication 2 due to severe necrosis and wilting of *B. pilosa* treatment seelings. Treatments were statistically compared with *L. sativa,* independently for each date and separately for each replication, using completely randomized design analyses.

Replications were compared for leaf area or plant height at seven days after seeding for *L. sativa* and 14 days after seeding for other species, using Student's *t* test, to determine if increased *B. pilosa* root density enhanced root exudate-induced inhibition of seedling growth. Resin control and *B. pilosa* treatments, expressed as percent of the pot control, were computed for each species to show differences in sensitivity of acceptor species to the allelochemicals. Average dry weight was measured for the eight seedlings of each species × treatment combination on the last day data were taken on leaf area or plant height.

## RESULTS

*B. pilosa* root exudates significantly inhibited growth of acceptor seedlings (Figures 2–5). *L. sativa* was the species most inhibited, followed by *P. vulgaris,* while *Z. mays* and *S. bicolor* were the least inhibited (Table 1). Severe necrosis and wilting of *L. sativa* seedlings was followed by death and appeared to involve phytotoxicity, not pathogenic disease. The pot and resin controls were not affected. Differences between the pot control and resin control in replication 1 with *L. sativa* lessened toward the end of the experiment (10 days after seedling) (Figure 2). *L. sativa* seedlings were also severely inhibited during replication 2 (Table 2), and plants died between the eighth and tenth day after seeding during replication 2.

*P. vulgaris* showed a clear separation in leaf area between all treatments beginning the sixth day after seeding (Figure 3). The differences between treatments increased with time.

TABLE 1. SPECIES VARIATION IN SENSITIVITY TO ALLELOPATHIC ROOT EXUDATES OF *Bidens pilosa* L.[a]

| | Seedling height or leaf area as % of pot control | | | |
|---|---|---|---|---|
| Treatment | *L. sativa* | *P. vulgaris* | *Z. mays* | *S. bicolor* |
| Resin control | 95.8 | 81.5 | 69.2 | 87.3 |
| *B. pilosa* | 36.4 | 56.4 | 62.4 | 70.9 |

[a]For *L. sativa* and *P. vulgaris,* leaf areas were measured; for *Z. mays* and *S. bicolor,* seedling height. Values for all crop species except *L. sativa* represent an average of two replications of eight seedlings at 11 days after seeding. Values for *L. sativa* represent an average of eight seedlings at 11 days after seeding from replication 1 only.

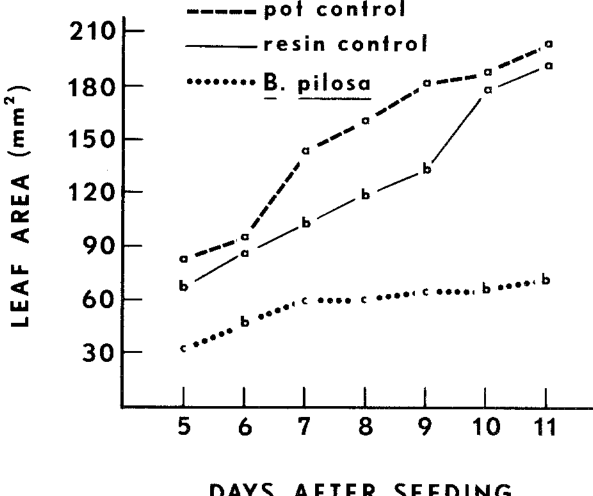


FIG. 2. Inhibition of *Lactuca sativa* L. seedling growth by *Bidens pilosa* L. root exudates. Points represent an average of eight seedlings from replication 1 only. Means (points) within days after seeding, represented by the same letter, are not significantly different at the 0.01 level (Student-Newman-Keuls' test).

*S. bicolor* (Figure 4) responded much the same as *P. vulgaris*. Differences in plant height between the three treatments were significant at the 0.01 level at each date beginning the seventh day after seeding, and the separations increased with time. The pot control experienced a rapid increase in growth, while the *B. pilosa* treatment ceased growth on the thirteenth day after seeding.

*Z. mays* did not show significant differences in plant height between treatments until the eighth day after seeding (Figure 5). Then differences between the pot control and the other treatments were highly significant. No differences existed between *B. pilosa* with XAD-4 and *B. pilosa* without XAD-4.

TABLE 2. INHIBITION OF *Lactuca sativa* L. SEEDLING GROWTH BY *Bidens pilosa* L. ROOT EXUDATES DURING REPLICATION 2[a]

| | *L. sativa* leaf area (mm²) at days after seeding | | |
|---|---|---|---|
| Treatment | 5 | 6 | 7 |
| Pot control | 81.1a | 111.2a | 140.8a |
| Resin control | 64.6b | 85.6b | 117.6b |
| *B. pilosa* | 31.0c | 32.8c | 36.7c |

[a] Means, within columns, followed by the same letter are not significantly different at the 0.01 level (Student-Newman-Keuls' test).

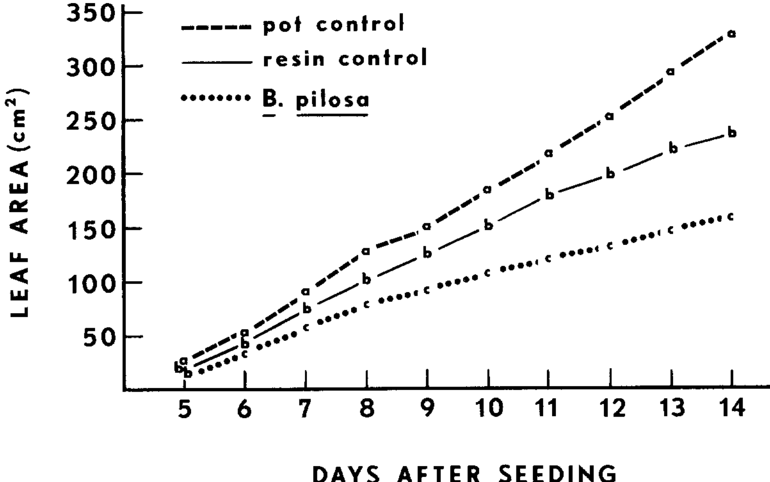


FIG. 3. Inhibition of *Phaseolus vulgaris* L. seedling growth by *Bidens pilosa* L. root exudates. Points represent an average of two replications of eight seedlings. Means (points) within days after seeding, represented by the same letter, are not significantly different at the 0.01 level (Student-Newman-Keuls' test).

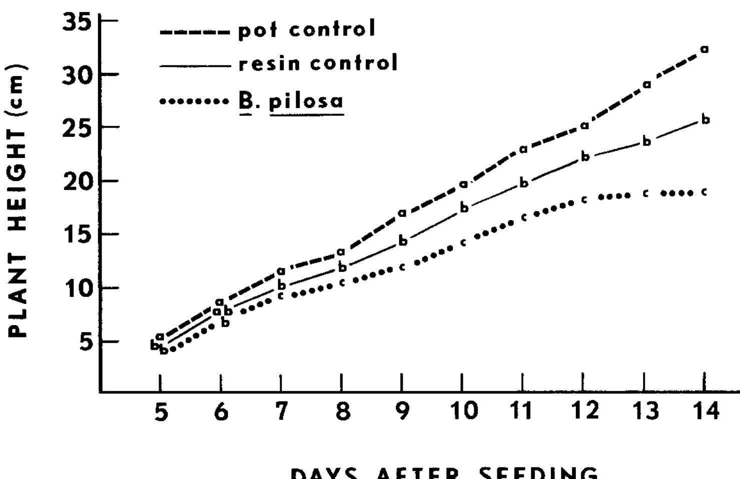


FIG. 4. Inhibition of *Sorghum bicolor* (L.) Moench seedling growth by *Bidens pilosa* L. root exudates. Points represent an average of two replications of eight seedlings. Means (points) within days after seeding, represented by the same letter, are not significantly different at the 0.01 level (Student-Newman-Keuls' test).

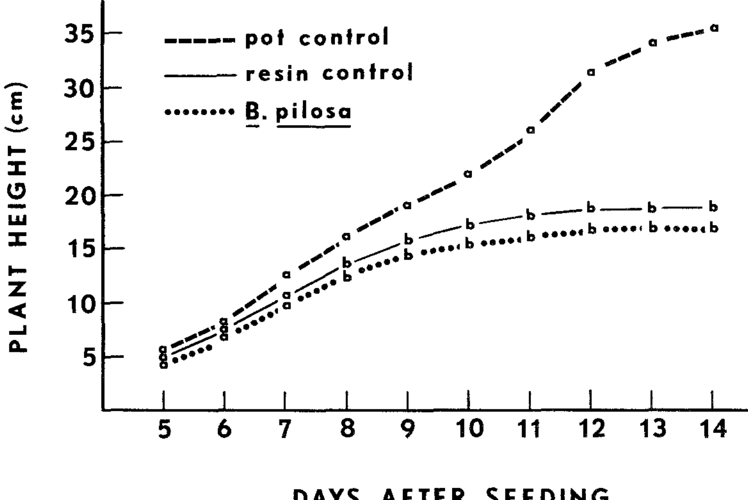


FIG. 5. Inhibition of *Zea mays* L. seedling growth by *Bidens pilosa* L. root exudates. Points represent an average of two replications of eight seedlings. Means (points) within days after seeding, represented by the same letter, are not significantly different at the 0.01 level (Student-Newman-Keuls' test).

Dry weight results followed plant height or leaf area results closely, except for *Z. mays* which showed greater difference between the resin control and the *B. pilosa* treatment with dry weight than with plant height (Table 3). Differences between replications varied with acceptor species (Table 4). *P. vulgaris* leaf area was significantly larger at the end of replication 1 than replication 2 with the *B. pilosa* treatment. The reverse occurred with the resin control, and no significant differences were found with the pot control. The only treatment with *L. sativa* that had differences due to replication was *B. pilosa.* Replication 2 was more

TABLE 3. EFFECTS OF *Bidens pilosa* L. ROOT EXUDATES ON DRY WEIGHT OF CROP SEEDLINGS[a]

| Treatment | Average seedling dry weight (mg) | | | |
|---|---|---|---|---|
| | *L. sativa* | *P. vulgaris* | *Z. mays* | *S. bicolor* |
| Pot control | 5.8 | 1603 | 372 | 194 |
| Resin control | 4.9 | 1272 | 204 | 136 |
| *B. pilosa* | 2.2 | 878 | 175 | 113 |

[a]Values for all crop species except *L. sativa* represent an average of two replications of eight seedlings at 14 days after seeding. Values for *L. sativa* represent an average of eight seedlings at 11 days after seeding from replication 1 only.

TABLE 4. INFLUENCE OF SIZE (AGE) OF *Bidens pilosa* L. PLANTS ON INHIBITION OF CROP SEEDLING GROWTH[a]

| | Seedling size | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *L. sativa* | | *P. vulgaris* | | *Z. mays* | | *S. bicolor* | |
| Treatment | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 |
| Pot control | 142.5 | 140.8 | 308.6 | 343.5 | 34.8 | 36.1 | 28.6 | 35.5** |
| Resin control | 102.9 | 117.6 | 219.0 | 246.8** | 17.3 | 20.7 | 23.4 | 27.9** |
| *B. pilosa* | 59.6 | 36.7** | 184.8 | 132.8** | 16.7 | 18.4 | 16.3 | 21.8** |

[a] *B. pilosa* plant size compared using replication with time. Replication (Rep) 1 was started approximately 21 days and replication 2 approximately 49 days after transplanting *B. pilosa* to pots. Seedling size measured at 14 days after seeding as leaf area ($cm^2$) for *P. vulgaris*, and plant height (cm) for *Z. mays* and *S. bicolor*. *L. sativa* seedling size measured at seven days after seeding as leaf area ($mm^2$). Values given represent an average of eight seedlings. The (**) indicates significant differences between means of replications for specific treatments within crop species at the 0.01 level (Student's $t$ test).

inhibitory to seedling growth than replication 1. The *B. pilosa* treatment averaged 42% of the pot control at seven days after seeding for replication 1 compared to 26% of the pot control for replication 2. *Z. mays* did not show differences between replications. *S. bicolor* plants were taller during replication 2 regardless of treatment.

## DISCUSSION

All acceptor plant species showed highly significant differences between the pot controls and *B. pilosa,* and all except *Z. mays* had statistically significant differences between the *B. pilosa* treatment and the resin control. Since the major known difference between *B. pilosa* with and without XAD-4 was the presence or absence of hydrophobic organic compounds in the circulating nutrient solution, the results indicate allelopathic inhibition of *L. sativa, P. vulgaris,* and *S. bicolor* by the hydrophobic root exudates of *B. pilosa.*

The four acceptor species also showed differences between the resin control and the pot control. This could not have been caused by differential nutrient absorption by *B. pilosa* plants, as nutrient levels of all hydroponic solutions were monitored and maintained at close to 0.5-strength Hoagland's. It is also doubtful that the XAD-4 resin became saturated and hydrophobic substances escaped the columns. XAD-4 has a very high specific surface area (750 $m^2/g$), and recovery of model organic compounds such as alcohols, esters, phenols, ketones, aldehydes, and acids from water at 2–10 parts per billion is better than 80% (Junk et al., 1974; Tateda and Fritz, 1978). In view of the high adsorbent capacity of XAD-4 and because columns were changed every other day, it was assumed that hydrophobic compounds were entirely removed from the resin control. A more likely possibility would be the presence of hydrophilic root exudates which were allelopathic and nonadsorptive to the XAD-4 column. All seedling species except *L. sativa* were progressively inhibited of normal growth rate in the resin control toward the end of the experiment, suggesting an accumulation of the hydrophilic growth inhibitors. As shown in Figure 2, the *L. sativa* resin control grew nearly as rapidly as the pot control after the ninth day, suggesting that *L. sativa* was quite resistant to the hydrophilic inhibitors but highly susceptable to the hydrophobic compounds.

Response to the allelochemicals varied between acceptor species, and the two dicotyledons were more sensitive than the monocotyledons. Acceptor species differed in their ratio of response to compounds trapped by the XAD-4 column and compounds that bypassed the column. Differences between the resin control and the pot control were presumed to be the result of inhibition by hydrophilic root exudates, while differences between the resin control and *B. pilosa* were presumed to be caused by hydrophobic root exudates. Differences between *B. pilosa* and the pot control represent the total allelopathic effect. It

is possible that growth-promoting substances were also released, but the overall effect of the root exudates was inhibitory.

All acceptor species showed increased differences between the pot control and the *B. pilosa* treatment with time, and some acceptors ceased growth in response to the *B. pilosa* treatment toward the end of the experiment. Differences between the pot and resin controls also increased with time with *P. vulgaris* and *S. bicolor.* These responses were probably caused by the accumulation of allelopathic root exudates, as nutrient solutions were not changed during the experiment. Accumulation of root exudates in soil could occur under natural conditions, particularly in areas with sparse rainfall where leaching is minimal.

*L. sativa* appeared to be more sensitive to hydrophilic allelochemics as young seedlings up to nine days after seeding, causing significant differences between the pot control and the resin control at this time (Figure 2). Among the tested crops, *L. sativa* was the most sensitive to allelochemicals. When five crops species were tested for seedling radicle growth in response to extracts of several weed species, *L. sativa* was the most inhibited with 0% radicle growth 72 hr after planting (Gliessman, 1983). In our study, wilting that accompanied necrosis and death of seedlings exposed to the *B. pilosa* treatment may be similar to that of *Asclepias syriaca* when exposed to phenylheptatriyne extracted from *B. pilosa* leaves (Campbell et al., 1982). Because the resin control was free from these symptoms, they were caused by the hydrophobic root exudates.

*P. vulgaris* appeared to be affected by both hydrophobic and hydrophilic allelochemics, as indicated by the growth curve of the resin control being nearly equidistant between those of the pot control and *B. pilosa.*

*S. bicolor* was also affected by both hydrophilic and hydrophobic allelochemicals, and the response of this species was similar to that of *P. vulgaris.* However, toward the end of the experiment, seedlings exposed to the *B. pilosa* treatment ceased growth (Figure 4). *S. bicolor* appeared to be more sensitive to hydrophobic allelochemicals than *P. vulgaris,* as indicated by the greater differences between *B. pilosa* with and without XAD-4 at 12–14 days after seeding.

*Z. mays* was the most sensitive species to exudates that bypassed the XAD-4 column. Differences in plant height between *B. pilosa* with and without XAD-4 were not significant at any time, and both of these treatments stopped growing simultaneously beginning the eleventh day after seeding. Since the hydrophobic inhibitors were removed from the resin control, it would appear that *Z. mays,* unlike the other three testing species, was much more sensitive to hydrophilic than hydrophobic exudates. However, dry weight results did show response to hydrophobic allelochemicals, and this could be due to a reduction in root growth by the hydrophobic exudates.

The significant decrease in growth of replication 2 seedlings compared to replication 1 with *L. sativa* and *P. vulgaris* was probably caused by larger *B. pilosa* root systems and the resultant increase in allelopathic root exudates. These

results were as expected because *B. pilosa* plants were one month older during replication 2 and had larger root systems. The increased growth of replication 2 seedlings compared to replication 1 with the *P. vulgaris* resin control, and all treatments with *S. bicolor,* could be due to higher temperatures during replication 2. Greenhouse temperatures were generally higher during replication 2 than replication 1. Temperature may affect the response of *S. bicolor* to allelochemicals as reported by Einhellig and Eckrich (1984). In our case, *S. bicolor* seedlings grew faster under higher temperatures, regardless of increased levels of root exudates.

Because the root exudates severely reduced seedling height or leaf area, the mode of action of the allelochemicals may be inhibition of growth. The mechanism of growth inhibition is not well understood at present and was not addressed in this study. Results of previous work indicate that polyacetylene compounds such as phenylheptatriyne are important allelochemics produced by *B. pilosa* (Campbell et al., 1982; Wat el al., 1979) and that these compounds act as photosensitizers in plants (Wat et al., 1979). Certain polyacetylenes such as alpha-terthienyl may also inhibit photosynthesis and $CO_2$ fixation (Sinclair and Arnason, 1982).

Sesquiterpene lactones, triterpenes, certain flavonoids, and cyclitols are also important secondary metobolites ubiquitous among the Compositae (Heywood et al., 1977). Other secondary metabolites found in many composites include pentacyclic triterpene alcohols, coumarins, flavones, methylated flavonols, and derivatives of caffeic acid (Heywood, et al., 1977). These compounds often possess sufficient hydrophobicity and would be adhered by XAD-4. Allelopathic compounds with more hydrophilic activity that would not be effectively trapped include glycosides of secondary metabolites, amino acids, certain organic acids, and alcohols.

The virtue of the recirculating system is that it eliminates competition for light; nutrient levels and pH can be controlled; and there is no physical contact between donor and acceptor plants or disturbance of the root systems. Root exudates are accumulated and continuously recirculated to acceptor plants and are not altered by extraction precedures. All materials used were either inorganic or relatively innert (e.g., Teflon), eliminating sources of organic compounds other than the root systems and rhizospheric microorganisms. This system also allows comparison of responses to hydrophobic and hydrophilic root exudates by the removal of hydrophobic compounds with XAD-4. Hydrophobic substances may be eluted from XAD-4 and their biological and chemical natures studied.

The results of this experiment confirm that root exudates of *B. pilosa* are allelopathic to the growth of seedlings representing different families of important crops. Various crop species may respond differently to allelopathic root exudates. Because *B. pilosa* was more inhibitory to other dicotyledons than to

monocotyledons, perhaps higher plants are more sensitive to allelochemicals produced by plants closely related taxonomically.

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# INFLUENCE OF THE OLFACTORY SENSE UPON SMOLT TRANSFORMATION IN SALMONID FISHES

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**Abstract**—The effect of olfactory deprivation on the process of smolt transformation in Atlantic salmon (*Salmo salar* L.) was investigated. Salmon parr were rendered anosmic by heat-cauterizing in November, and, together with controls, size, purine deposition (silvering), and mortality were monitored throughout spring in individuals within sibling groups. In May the fish were exposed to seawater (3.2% salinity) to determine levels of smolting in anosmic fish compared with control fish. Olfactory deprivation was found to induce a negative effect on survival in both anosmic fish as well as controls when reared within the same rearing tank. A 54% survival was metered in anosmic fish as opposed to only 26% survival in control fish during the experimental period. The low survival rates observed are suggested to result from abnormal behavior in anosmic fish due to lack of proper olfactory stimuli. Within each strain, the anosmic fish were found shorter and displayed less silvering than their control fish at the end of the experimental period. Further, since control fish could withstand salinity exposure longer than anosmic fish, smolting is suggested to have been retarded among the anosmic fish. Olfactory influence on smolt transformation may be caused by conspecific pheromones secreted for the purpose of mediating effects of life-history patterns in salmonid fishes.

**Key Words**—Salmon, *Salmo salar*, olfaction, anosmia, pheromones, smolt transformation, growth.

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## INTRODUCTION

Smolt transformation in salmonid fishes is a complex annual phenomenon which prepares the stream-living parr for a saltwater habitat. The transformation process includes morphological, physiological, and behavioral changes, presumably initiated by endogenous timing programs and regulated via environmental parameters (Hoar, 1976; Lundqvist, 1983). The morphological changes include body silvering resulting from deposition of purines (Johnston and Eales, 1967), together with a lengthening of the body, resulting in a lower coefficient of condition (Hoar, 1939). An important result of the physiological changes is increased salinity tolerance (Huntsman and Hoar, 1939; Parry, 1960). The behavioral changes include a switching from positive to negative rheotaxis, together with an increased tendency for schooling (White and Huntsman, 1938; Arnold, 1974).

The size of the fish has been suggested as an important control factor for smolting (Allen, 1944; Johnston and Eales, 1970). In addition, environmental parameters such as photoperiods, temperature, and salinity of the water have been found experimentally to affect smolt transformation (Saunders and Henderson, 1970; Wedemeyer et al., 1980; Clarke et al., 1981; Johnston and Saunders, 1981). The morphological, physiological, and behavioral events that occur during smolting are mediated via the hypophyseal endocrine system (Hoar, 1965; Komourdjian and Idler, 1977). The hypothalamus controls the endocrine function of the hypophysis, as reviewed by Ball and Baker (1969).

The olfactory bulb in fishes has direct nervous connections with the preoptic nucleus in the hypothalamus (Sheldon, 1912; Holmgren, 1920). Electric stimulation of the olfactory tract in gold-fish has been demonstrated to induce nerve impulses in the preoptic nucleus (Kandel, 1964) and also to deplete stainable neurosecretory granules from preoptic nucleus cells as well as their axons (Jasinski et al., 1966). In addition, electrical stimulation of the olfactory mucosa in *Ophiocephalus punctatus* by Chandrasekhar and Chacko (1970) gave similar results in the hypothalamus. The degree of degranulation following olfactory mucosa stimulation was found to be directly proportional to the duration of the stimulation, and axons descending down the hypothalamohypophyseal tract were found to be affected.

The intimate connection between the olfactory organ and the hypothalamohypophyseal region in the brain indicate that olfactory stimuli may affect the endocrine system of fishes, as generally recognized in higher vertebrates (Breipohl, 1982). During their life cycle, anadromous salmonids undergo two distinct stages of events that are regulated via the endocrine system: smolt transformation and sexual maturation. A pilot experiment to determine any possible effects of the olfactory sense upon sexual maturation in Atlantic salmon (*Salmo salar* L.) has previously been reported (Stabell and Refstie, 1980). The aim of the

present study was to determine whether the olfactory sense would affect smolt transformation in salmonid fishes.

## METHODS AND MATERIALS

*Experimental Fish and Handling.* The experiment was performed at the Research Station for Salmonids, Sunndalsøra Unit, Norway, from November 1979 to May 1980. Six sibling groups of Atlantic salmon (*Salmo salar* L.), representing strains of hatchery families, were used in the experiment. The fishes were presmolt (age 0+) at the start of the experiment and were expected to undergo smolt transformation during the following spring.

Fish within each genetic group were freeze-branded with a specific position code, according to the method described by Refstie and Aulstad (1975), and divided into two lots consisting of 25 individuals each. The fish within each lot were individually marked by using 25 of 32 possible combinations resulting from a clipping of either or both of the maxillary bones (upper jaw) and the ventral fins, together with the adipose fin. One of the lots within each group was rendered anosmic by cauterizing the olfactory mucosa on both sides with a soldering iron at 320°C. The heat exposure time was approximately 2 sec for each nostril. The other lot within each group served as olfactorily untreated controls. All handling, marking and operations were performed under anesthesia (0.003% chlorbutanol).

The marking and the treatment of the fish was carried out on November 20, 1979, and all genetic groups, including both treatment lots within each group, were raised together in the same freshwater tank until May 20, 1980. Throughout the experimental period, the fish were exposed to standard hatchery conditions and were fed ad libitum on commercially available food pellets from an automatic feeder.

*Sampling of Data.* Fork length was measured to the nearest millimeter at the start and end of the experiment. Smolt stage was determined by visual inspection at the end of the experiment, by ranging each fish in a scale from one to five. Stage one represented fish with distinct parr marks without any visible body silvering, whereas stage five represented a totally silver-colored fish with all visible parr marks absent. Anosmicized fish were easily sorted from control fish six months after the cauterizing, due to lack, or closure, of the nose openings resulting from the heat treatment. Data from individuals which had survived the experimental period revealed statistics on size of the fish at the start of the experiment.

After measurement and inspection on May 20, all fish were transferred to a tank containing seawater (3.2% salinity). Mortality resulting from salinity exposure was then registered two to three times per day during a 72-hr period.

*Statistical Evaluation.* Mean value ($\bar{X}$) and standard deviation (SD) were calculated for the current parameters within all groups of fish. Nonparametric statistics were used for additional evaluation of the data (Siegel, 1956) by testing trends in mean values appearing between related lots among the genetic groups. Differences were considered statistically significant when $P$ values were found lower than a probability level of 5%. The probabilities given are all one-tailed. In the case where two independent samples were present, the Mann-Whitney U test was applied. When dealing with cases of two related samples, the sign test was used.

## RESULTS

The mean values ($\bar{X}$) in length of the fish at the start of the experiment, together with the SDs, are presented for each lot in Figure 1A. The group numbers (freeze brands) of each sibling strain are given along the abcissa. The black bars represent the average initial values in length, given for all 25 fish within each lot. The black bars on the left-hand side within each group represent the fish rendered anosmic, the black bars on the right-hand side represent the control fish. The crossed-hatched bars within each group give the initial lengths of the anosmicized fish which survived the experimental period. The stippled bars within each group represent the initial lengths of the surviving control fish. Note that in group 46, only one control fish survived the experimental period.

As can be seen from the black bars in Figure 1A, the mean initial lengths of the anosmicized fish (left black bars) vary slightly from the mean initial lengths of the control fish (right black bars) within each strain. In total, a 0.08-cm shorter mean length was registered for all the anosmicized fish compared to all the control fish at the start of the experiment. The variations observed, however, reveal no statistically significant differences (sign test, $P = 0.188$).

However, among the fish which survived the experimental period, a significant difference in mean length occurred initially between the anosmicized fish (Figure 1A, cross-hatched bars) and the control fish (Figure 1A, stippled bars); the anosmicized fish being the larger (sign test, $P = 0.031$). In total, the anosmicized fish which survived the experimental period averaged 0.22 greater in mean length compared to the control fish at the start of the experiment, being 11.52 cm and 11.30 cm, respectively.

Figure 1B describes the relationship between surviving fish within each lot and the entire lot of 25 similarly treated individuals, expressed as percentage deviation in mean length at the start of the experiment. As can be seen in Figure 1B, the surviving anosmicized fish demonstrate a mean length at the start which deviates from their entire lot mainly in a positive direction; i.e., among the anosmicized fish it seems as if the larger individuals have survived the experimental period. Among the control fish, however, a negative deviation occurs

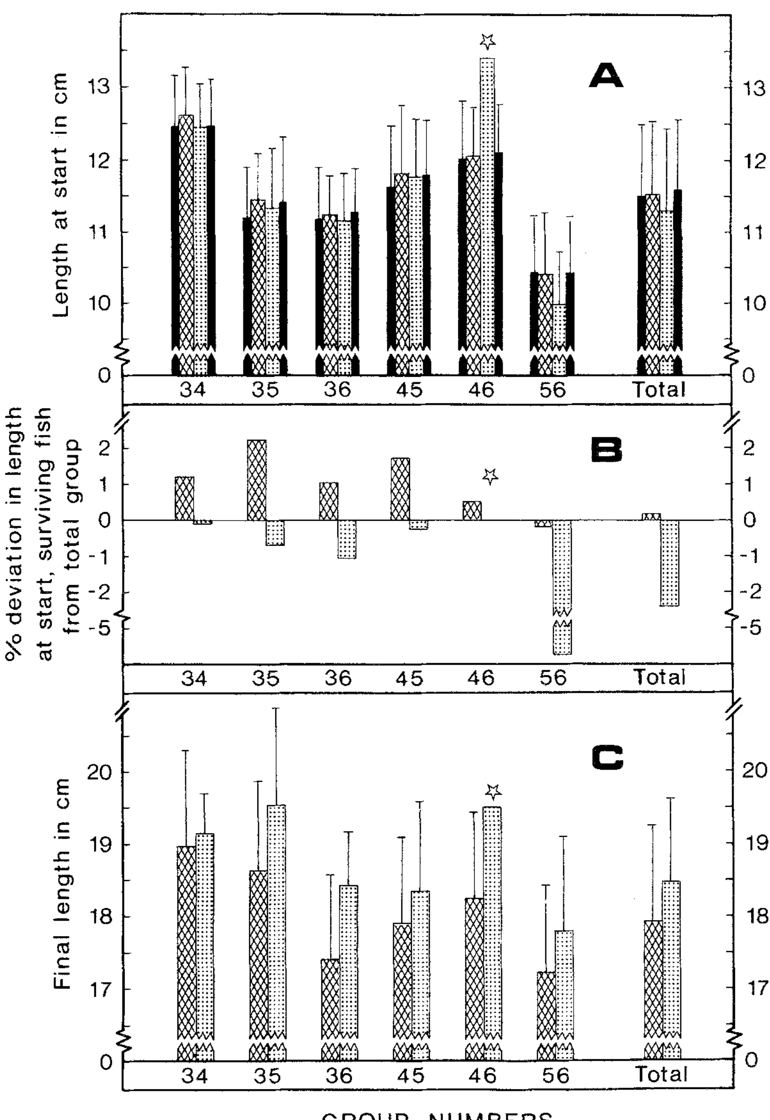


FIG. 1. Size distribution within groups of Atlantic salmon parr and presmolt given as mean values together with standard deviations, at start of the experiment in December (A and B) and at termination in May (C). Group numbers are equivalent to position codes from freeze branding (Refstie and Aulstad, 1975) and refer to sibling strains of fish. Hatched bars represent lots of fish within each genetic group rendered anosmic by heat cauterizing at start; stippled bars represent olfactorily untreated control lots. Within each lot the fish were individually marked by clipping of maxillary bones (upper jaw), and ventral or adipose fins in combinations. Results from the start of the experiment (A and B: hatched and stippled bars) were obtained from data on individuals which survived the experimental period. Black bars (A) represent data from total lots of 25 individuals at start, anosmicized fish on left-hand side within the groups, reference fish on right-hand side. Star indicates data from one fish.

with regard to mean length at the start of surviving individuals respective to their entire lot. In other words, among the control fish, the smaller individuals seem to have survived the experimental period. The trend revealed in Figure 1B, is statistically highly significant (Mann-Whitney U test, $P = 0.004$).

The mean lengths at the end of the experimental period, for both lots of fish within each genetic group, are presented in Figure 1C. The data reveal that the mean lengths of the anosmicized fish on May 20 were shorter than the mean lengths of the control fish within all sibling groups. The final mean lengths of the anosmicized fish averaged 17.93 cm, while the final mean lengths of the control fish averaged 18.47 cm. Among the genetic groups where SDs can be given for both treatment lots, the mentioned trend is statistically significant (sign test, $P = 0.031$).

Figure 2A presents the percent increase in mean length during the six-month experimental period. The data demonstrate that during that period, the control fish within each sibling group increased in length more than the anosmicized fish. It should be noted, however, that some anosmic lots (i.e., groups 35 and 56) increased their length more than control lots within other strains, suggesting genetic influence as being a more important factor than olfactory influence for growth. In total, the anosmicized fish increased their mean length by 56.4%, while the control fish increased by 64.5%, revealing a 8.2% larger mean length increase in the control fish. The trend dominates within all five groups where sufficient data have been obtained and occurs at a statistically significant level (sign test, $P = 0.031$).

Stage of smolting among the current lots on May 20 is presented in Figure 2B. All lots were well above the minimum size for smoltification of Atlantic salmon (Johnston and Eales, 1970). Again the data demonstrate a specific trend with regard to differences between mean values, revealing the control fish as systematically having the largest scores within the groups. In total, the control fish demonstrate a mean value in smolt stage of 3.6, while the anosmicized fish demonstrate a mean value in smolt stage of 3.5. This trend occurs at a significant statistical level (sign test, $P = 0.031$).

Figure 2C presents the survival of the fish during the experimental period as percentage survival within each lot from the entire and related lot. The data demonstrate that, within all groups, the anosmicized fish have survived the experimental period better than the control fish. In total, 54% of the anosmicized fish survived the experimental period, as opposed to only 26% of the control fish. The trend in survival within the groups is present at a statistically significant level (sign test, $P = 0.016$).

From the fish which survived the six-month period of the experiment, mortality resulting from subsequent salinity exposure is presented in Figure 3. During the 72 hr when salinity exposure was monitored, the mortality curve for the control fish was delayed at an approximate average of 6 hr compared to the

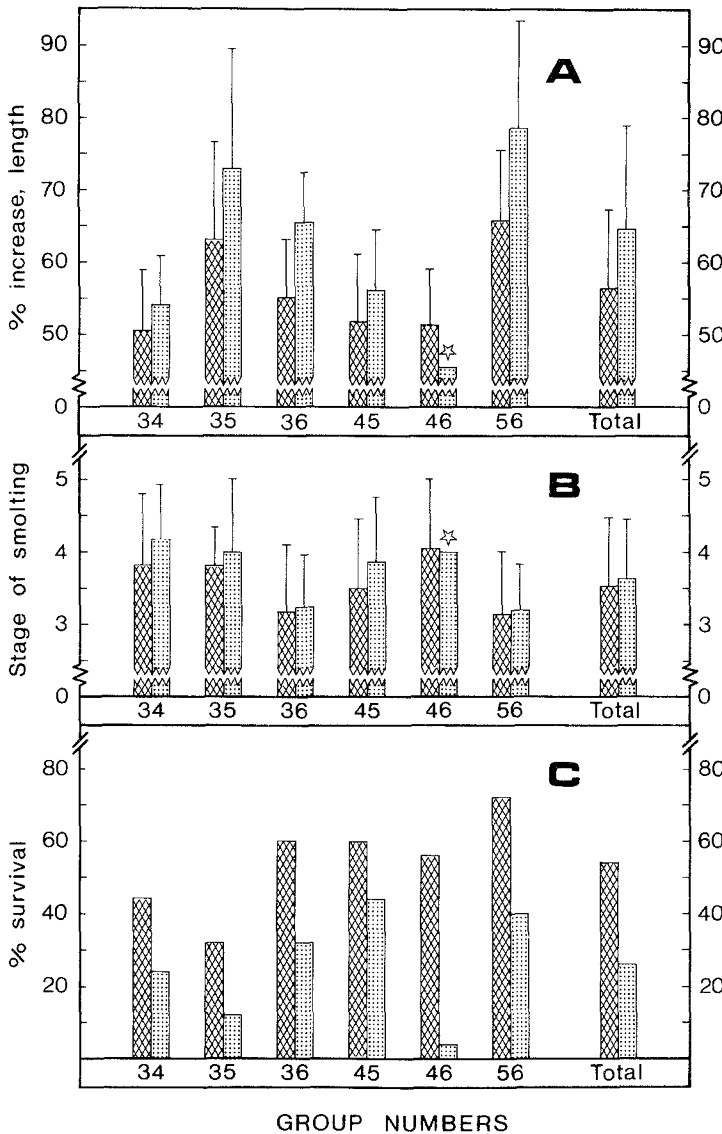


FIG. 2. Size increase during experimental period (A), together with stage of smolting (B) and percent survival (C) at the end of the experimental period within groups of Atlantic salmon presmolt. Data are given as mean values together with standard deviations. Group numbers are equivalent to those given in Figure 1. Hatched bars represent lots of fish within each genetic group rendered anosmic by heat-cauterizing at start; stippled bars represent olfactorily untreated control lots. Star indicates data from one fish.

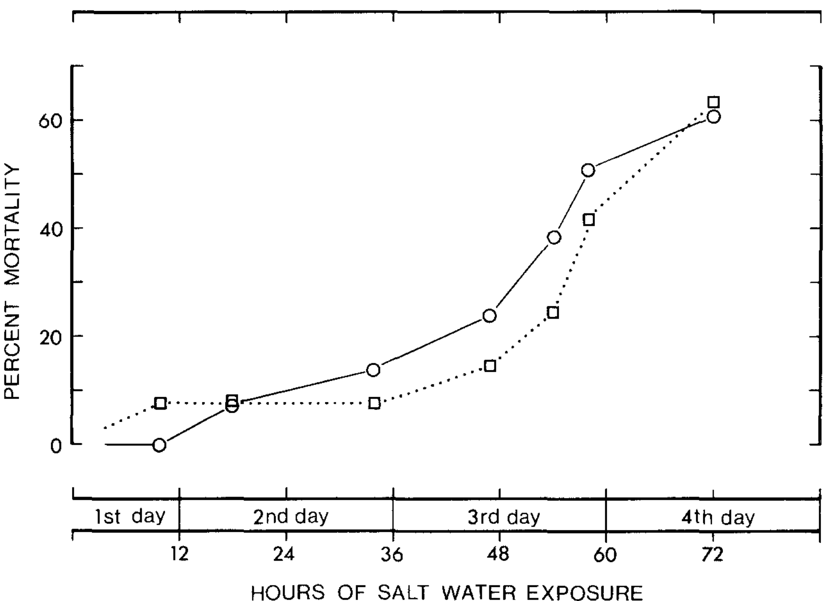


FIG. 3. Mortality of Atlantic salmon presmolt following seawater exposure (3.2% salinity) in May. Circles: fish rendered anosmic as parr in December. Squares: olfactorily untreated control fish.

mortality curve of the anosmicized fish. The data demonstrate that the control fish could better withstand salinity exposure than the anosmicized fish, indicating that smolting in the control fish was more advanced.

## DISCUSSION

The present study has been performed to investigate smolt transformation in salmonid fishes with special regard to the olfactory sense. The data obtained demonstrate that in addition to smolt transformation, population dynamics should also be considered. The "mass physiology" (e.g., Allee et al., 1940) revealed appears to incorporate effects relevant for both laboratory conditions as well as natural environments.

Survival of the anosmicized fish compared with survival of the control fish during the six months of investigation indicate that the process of cauterizing as such, performed to produce anosmic fish, does not interfere with survival rate. The result is in accordance with survival data from earlier experiments, following similar heat treatment (Stabell and Refstie, 1980). The experiment took place under standard hatchery conditions. Since the control lots of fish consisted of randomly sampled individuals taken from the same sibling groups and rearing

tanks as their anosmicized counterparts, the low survival rate of control fish during the experimental period cannot be attributed to any treatment effects. In consequence, the results obtained in the present study should be related to the fact that the cauterized fish had been denied olfaction.

It appears also that lack of the olfactory sense did not interfere with food intake, since final length and length increase (i.e., growth) were both found to be substantial among some anosmic lots. In addition, the data on final length and length increase did not reveal any consistent differences between anosmic fish and control fish. Significant differences between the treatment types were found only when systematic variations within family strains were considered. It appears, therefore, that the results obtained should be related to aspects of olfactorily modified growth rather than to olfactory aspects of food intake. The conclusion concerning olfaction and food intake are in accordance with current knowledge on feeding behavior and physiology of salmonids. Salmonid fishes are predominantly sight-feeders and chemical feeding stimulants do not seem to be detected before their appearance in the oral cavity (Mackie, 1982).

A population of salmon displays large variations in size among individuals within year classes (Eriksson et al., 1979). Accordingly, to avoid masking effects from random sampling, studies in aspects of salmonid physiology related to size should be based on data from individual fish. When treated statistically, the diversity in size found within salmon populations will necessarily result in large SDs, pointing to parametric statistics as being inadequate for evaluating trends indicated in the data. In the current study, therefore, evaluation of data has been performed by nonparametric statistics, focusing upon trends in mean values for measured parameters appearing between treatment lots within strains of fish.

Among the anosmicized fish, those of an initial larger size survived the experimental period, while those of a smaller size survived among the control fish. If olfactory stimulants secreted by a fish population induce hierarchic behavior, the observed event could result from an inability of the anosmic fish to perceive the regulating stimulus. That is, behavior of anosmic fish may have been adjusted in accordance with the lack of olfactory sensation perceived, and the result obtained may reflect dominance behavior in a fish rearing tank. Potential dominant individuals among the intact fish, and possibly also the smaller anosmic individuals, may then have been exposed to an increased level of stress induced by the larger anosmic individuals not behaving "normally." In a population of young salmon, the fastest growing parr are thought to be the first to become smolts (Allen, 1944; Jones, 1959; Parry, 1960). During smolting, salmonids are very sensitive to stress (Schreck, 1982). An additional argument, apparently supported by the low survival rate observed among the initially larger control fish during the experimental period, could be that the most advanced level of smolting was present among individuals which had the larger size at start.

Purine depositions during parr–smolt transformation have been found to be dependent on body size (Johnston and Eales, 1970). In addition, a tendency has been found for those smolts within a year class which were largest at the end of the winter to migrate first (Allen, 1944). Growth made in spring, however, tended to make the size of the smolt more uniform at migration.

In the current study, the genetic groups having the smallest size at the start (i.e., groups 35, 36, and 56) demonstrated the largest relative increase in length during spring. This result points to inheritance as more important than olfaction as regards growth. The visually observed smolt stages, however, seem related to final length and not to a relative increase in length during spring. Smolt size has been reported to be in the range of 12.5–15.5 cm (Hoar, 1939; Allen, 1944; Johnston and Eales, 1970), while the average fish lengths within the groups of the current study were all found to exceed 17 cm at the end of the experiment. The data presented, therefore, seem to fulfill the size requirements reported as essential for Atlantic salmon to undergo parr–smolt transformation.

The anosmic lots within the current strains all demonstrated an average shorter length than their control lots at the end of the experimental period. In addition, purine deposition was found to be less developed in the anosmic lots. Together with the fact that the control fish could withstand salinity exposure longer, satisfactory evidence seems to exist for smolt transformation being less developed in the anosmic lots compared to their control lots of fish. It should be stressed that smolting was not completed in any group or treatment lot at the time of salinity exposure. However, since a more pronounced effect was to be expected before smolting was fully completed (i.e., Clarke and Blackburn, 1977), salt water exposure was performed at the chosen date.

Allee et al. (1940) demonstrated an effect from homotypically conditioned water on growth in individually raised goldfish. The authors, however, gave no evaluation of sensory pathways by which the effect from chemical signals could be mediated in the receiving fish. The differences in final length observed between experimental lots in the current study may well result from growth effects similar to those observed by Allee et al. (1940). If so, the effect observed must result from conspecific chemical stimuli not being perceived by the anosmic fish. Consequently, the signal effect must be mediated via the olfactory system and must indirectly affect smolt transformation. The differences in length obtained in the current study, however, could well be explained from lack of olfactory stimuli affecting the endocrine process of smolt transformation directly, resulting in an incomplete lengthening of the body (Hoar, 1939). This last suggested type of olfactory-regulated growth should also include the potential influence from conspecific chemical signals. Therefore, both suggested alternatives for olfactorily mediated growth effects will apparently affect life history patterns of those salmonid species which undergo smolt transformation.

We conclude that the data obtained in the current study may result either from a lack of proper olfactory stimuli affecting the process of smolt transfor-

mation directly or from an indirect effect on smolt transformation mediated via a lack of proper and superimposed olfactory influence on growth in general. Whatever the physiological pathways involved, we find it reasonable to propose that the observed results are due to lack of sensory input from odorants secreted by conspecifics (i.e., pheromones) for the purpose of mediating effects on life history patterns within populations of fish.

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# INSECT ANTIFEEDANT ACTIVITY OF CLERODANE DITERPENOIDS AGAINST LARVAE OF *Spodoptera Littoralis* (BOISD.) (LEPIDOPTERA)

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**Abstract**—Antifeedant activities of nine clerodane diterpenoids, isolated in this laboratory from different species of *Ajuga* plants, have been studied against larvae of Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.) (Lepidoptera) by application of the leaf disk method. Evaluation of activity was carried out by calculating, at different time intervals, the feeding ratio (FR) from the relationship between the consumed areas of treated disks (CTD) and control disks (CCD); for comparison purposes a $FR_{50}$ defined as the FR at a CCD of 50% was established. Some compounds exhibited activity at a 0.01 $\mu g/cm^2$ dose (0.3 ppm). Structure–activity relationships are discussed

**Key Words**—Insect antifeedant, *Spodoptera littoralis* (Boisd.), Lepidoptera, Noctuidae, Clerodane diterpenoids, *Ajuga* plants.

## INTRODUCTION

Considerable attention has been recently paid to the insect antifeedant activities of some natural clerodane diterpenoids isolated from different plant families (Hanson, 1979). This activity has been associated to definite structural features of these compounds. In this context, on the basis of activities exhibited by perhydrofuro [2, 3-b] furan analogs I a–d, Kojima and Kato (1981) pointed out that this moiety might be the responsible site for activity in the clerodane structure. However, this statement was in disagreement with the high feeding inhibition against *Spodoptera littoralis* and *S. exempta*, elicited by ajugarin II (Kubo et al., 1976), in which that moiety is replaced by a butenolide ring.

On the other hand, different authors (Jackson and Ley, 1981; Luteijm and de Groot, 1981; Geuskens et al., 1983) related the antifeedant activity of cler-



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odanes to the presence of a decalin ring system bearing one spiroepoxide substituent at C-4 and two acetate groups at C-6 and C-18. Several synthetic analogs, designed to prove these hypotheses, have failed to evoke similar responses as those elicited by naturally compounds on various insects. Thus, analogs III and IV induced feeding inhibition in *Pieris brassicae*, but they were unsuccessful in a polyphagous insect such as *Spodoptera* (Geuskens et al, 1983), whereas analog V exhibited moderate activity against *Locusta migratoria* (Ley et al., 1981).

From the above results, it has been suggested that the antifeedant activity of clerodanes on *Spodoptera* species might be related to a synergistic action of the furofuran ring and the epoxy diacetate groups of the *trans* decalin moiety. (Geuskens et al., 1983). To clarify this point, in the present communication, we report the results of the study of antifeeding activities of selected natural clerodane diterpenoids, isolated from Mediterranean *Ajuga* plants, and of some derivatives thereof, as well as of some simple synthetic butenolide derivatives, prepared in this laboratory (cf. Fig. 1). These activities have been evaluated by application of the choice test method, using *S. littoralis* larvae, described by Bellés and Piulachs (1983).

## METHODS AND MATERIALS

*Insects.* Newly ecdysed fifth-instar larvae of *S. littoralis*, collected from a laboratory culture, reared on a semiartificial diet (Poitout and Bues, 1974), at 25 ± 2°C, 60–70 relative humidity and 18 hr of photophase, were used for antifeeding bioassays.

*Compounds.* Ajugareptansin (VI) and ajugareptansone A (VII) were isolated from *Ajuga reptans* (Camps et al., 1979, 1981), whereas pentaol VIII was obtained by reduction of VI with lithium aluminium hydride in dry diethyl ether. Ivains IX–XII were isolated from *A. iva* (Camps et al., 1982), whereas 2-acetylivain 1 (XIII) and 14,15-dihydroajugapitin (XVIII) were found in *A. pseudoiva* (Camps et al., 1984a). Compound XIII was also prepared by acetylation of parent compound IX with acetic anhydride and pyridine. Ajugapitin (XIV) and its derivatives XV and XVI were isolated from *A. chamaepitys* (Hernandez et al., 1982; Camps et al., 1984a,b), whereas 2-acetyl-14,15-dihydroajugapitin (XVII) was obtained by acetylation of compound XVIII under the above conditions, and 14-hydroajugapitin-2,15-dione (XIX) was prepared by oxidation of compound XVI with chromium trioxide in pyridine (Camps et al., 1984b). $\gamma$-Butenolide derivatives XX–XXIII were synthesized in our laboratory by conventional procedures (J. Caixach, F. Camps and J. Coll, unpublished results).

*Antifeeding Bioassays.* Choice tests were carried out using leaf disks of lettuce, *Lactuca sativa* with an area of 1 cm$^2$ and an average weight $\overline{X} = 33.65 \pm 0.07$ mg ($N = 10$). Compounds to be tested were uniformly distributed on

the upper surface of the disks by application of 10 $\mu$l acetone solutions with a microsyringe followed by evaporation of the solvent (treated disks: TD). Control disks (CD) were analogously treated with acetone. In each bioassay, four treated and four control disks were alternatively placed in a covered polyethylene Petri dish (8.5 cm diameter) in the presence of five larvae. Experiments were performed under the same temperature and humidity conditions of the laboratory culture, but in constant darkness.

*Evaluation Procedure.* Consumed areas of treated disks (CTD) and those of controls disks (CCD) were simultaneously measured at regular intervals; usually every 30 min, during 4–5 h, to calculate the corresponding feeding ratio FR = CTD/CCD. For comparative purposes, we recommend the use of $FR_{50}$, i.e., the ratio when 50% of CD areas has been consumed ($CCD_{50}$). These values can be easily obtained in each case by extrapolation of the nearest empirical values.

## RESULTS AND DISCUSSION

As shown in Table 1, most of the natural products studied exhibited good to excellent antifeeding activities ($FR_{50} < 0.5$) at 1-$\mu$ g doses ($\simeq$30 ppm), with the remarkable exception of ajugareptansin (VI) and ajugareptansone A (VII). The low activity of these compounds might be presumably accounted for by conformational modifications, as inferred from X-ray diffraction studies of ajugareptansin *p*-bromobenzoate (Solans et al., 1979). In this molecule, ring A of the decalin system displays a skew boat conformation, probably due to the steric hindrance between substituents at C-1 and C-9. This hindrance might also have a definite influence on the free rotation of the hexahydrofurofuran system that has been considered important for the activity of clerodane diterpenoids (Kojima and Kato, 1981).

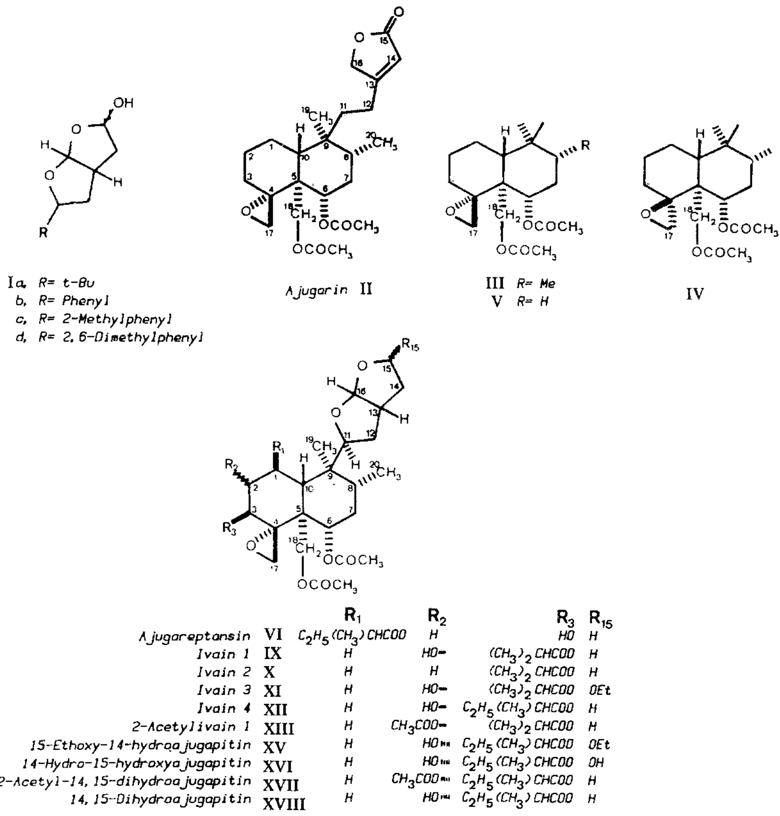
The most active compounds were found in the ajugapitin series with activities 10- to 100-fold higher than those shown by the ivain group. For comparison purposes between both series, taking ivain 2 (X) as the parent structure, replacement of an $\alpha$ hydrogen at C-2 by a hydroxy group increases 10-fold the activity (cf X and XVIII), whereas the replacement of the corresponding epimer hydrogen by the same group diminishes it to an equivalent extent (cf. X and IX). On the other hand, substitution of acetoxy groups for those hydrogen atoms has no significant effect in the activity of the $\alpha$-epimer (cf. X and XVII) and shows approximately a 10-fold decrease in the activity of the $\beta$-epimer (cf. X and XIII).

Apparently, the nature of the acyl radical of the ester moiety at C-3 has no influence on the activity (cf. IX and XII). Substitution of hydroxy or ethoxy groups for hydrogen at C-15 in the hexahydrofurofuran moiety decreases the activity in the ajugapitin series (cf. XVIII and XVI or XV) whereas the same

TABLE 1. FEEDING RATIOS OF TEST COMPOUNDS.[a]

| Compound | Dose (μg/cm²) | N | $FR_{50} \pm$ SEM | $FR_{75} \pm$ SEM |
|---|---|---|---|---|
| VI | 10 | 5 | 0.20 ± 0.07 | 0.26 ± 0.11 |
| | 1 | 5 | 0.60 ± 0.10 | 0.72 ± 0.18 |
| VII | 25 | 5 | 0.74 ± 0.12 | 1.01 ± 0.13 |
| | 10 | 5 | 1.15 ± 0.08 | 1.10 ± 0.07 |
| VIII | 25 | 3 | 0.67 ± 0.18 | 0.85 ± 0.31 |
| | 10 | 4 | 0.99 ± 0.23 | 1.22 ± 0.37 |
| IX | 1 | 5 | 0.25 ± 0.07 | 0.52 ± 0.09 |
| | 0.1 | 5 | 0.74 ± 0.13 | 0.92 ± 0.09 |
| X | 1 | 5 | 0.14 ± 0.02 | 0.16 ± 0.02 |
| | 0.1 | 5 | 0.25 ± 0.09 | 0.29 ± 0.10 |
| | 0.01 | 7 | 0.67 ± 0.10 | 0.74 ± 0.09 |
| XI | 1 | 5 | 0.37 ± 0.10 | 0.42 ± 0.13 |
| | 0.1 | 5 | 0.71 ± 0.13 | 0.79 ± 0.13 |
| XII | 1 | 5 | 0.12 ± 0.04 | 0.13 ± 0.03 |
| | 0.1 | 5 | 0.70 ± 0.13 | 0.87 ± 0.06 |
| XIII | 1 | 5 | 0.12 ± 0.04 | 0.14 ± 0.03 |
| | 0.1 | 5 | 0.51 ± 0.05 | 0.51 ± 0.06 |
| XIV | 1 | 5 | 0.04 ± 0.00 | 0.04 ± 0.00 |
| | 0.1 | 5 | 0.24 ± 0.06 | 0.31 ± 0.08 |
| | 0.01 | 5 | 0.41 ± 0.10 | 0.39 ± 0.08 |
| | 0.001 | 3 | 0.74 ± 0.05 | 0.54 ± 0.09 |
| XV | 10 | 6 | 0.11 ± 0.03 | 0.28 ± 0.07 |
| | 1 | 6 | 0.39 ± 0.07 | 0.52 ± 0.10 |
| XVI | 1 | 10 | 0.30 ± 0.09 | 0.31 ± 0.09 |
| | 0.1 | 4 | 0.37 ± 0.08 | 0.91 ± 0.18 |
| XVII | 1 | 4 | 0.06 ± 0.02 | 0.08 ± 0.02 |
| | 0.1 | 4 | 0.52 ± 0.06 | 0.64 ± 0.08 |
| | 0.01 | 4 | 0.62 ± 0.12 | 0.80 ± 0.11 |
| XVIII | 1 | 5 | 0.11 ± 0.04 | 0.11 ± 0.04 |
| | 0.1 | 5 | 0.22 ± 0.08 | 0.27 ± 0.09 |
| | 0.01 | 5 | 0.32 ± 0.10 | 0.32 ± 0.07 |
| | 0.001 | 3 | 0.46 ± 0.20 | 0.58 ± 0.25 |
| XIX | 25 | 4 | 0.56 ± 0.09 | 0.70 ± 0.20 |
| | 10 | 4 | 0.48 ± 0.07 | 0.72 ± 0.11 |
| XX | 25 | 2 | 0.23 ± 0.03 | 0.25 ± 0.03 |
| | 10 | 4 | 0.72 ± 0.07 | 0.78 ± 0.11 |
| XXI | 25 | 3 | 0.10 ± 0.02 | 0.10 ± 0.02 |
| | 10 | 4 | 0.77 ± 0.18 | 0.67 ± 0.15 |
| XXII | 50 | 2 | 0.39 ± 0.15 | 0.60 ± 0.01 |
| | 25 | 6 | 0.70 ± 0.11 | 0.69 ± 0.10 |
| XXIII | 50 | 2 | 0.80 ± 0.17 | 1.22 ± 0.07 |
| | 25 | 2 | 1.34 ± 0.46 | 0.9 ± 0.30 |

[a] Structures of these and other compounds appear in Figure 1.




| | | $R_1$ | $R_2$ | $R_3$ | $R_{15}$ |
|---|---|---|---|---|---|
| Ajugareptansin | VI | $C_2H_5(CH_3)CHCOO$ | H | HO | H |
| Ivain 1 | IX | H | HO– | $(CH_3)_2CHCOO$ | H |
| Ivain 2 | X | H | H | $(CH_3)_2CHCOO$ | H |
| Ivain 3 | XI | H | HO– | $(CH_3)_2CHCOO$ | OEt |
| Ivain 4 | XII | H | HO– | $C_2H_5(CH_3)CHCOO$ | H |
| 2-Acetylivain 1 | XIII | H | $CH_3COO$– | $(CH_3)_2CHCOO$ | H |
| 15-Ethoxy-14-hydroajugapitin | XV | H | HO''' | $C_2H_5(CH_3)CHCOO$ | OEt |
| 14-Hydro-15-hydroxyajugapitin | XVI | H | HO''' | $C_2H_5(CH_3)CHCOO$ | OH |
| 2-Acetyl-14,15-dihydroajugapitin | XVII | H | $CH_3COO$''' | $C_2H_5(CH_3)CHCOO$ | H |
| 14,15-Dihydroajugapitin | XVIII | H | HO''' | $C_2H_5(CH_3)CHCOO$ | H |

FIG. 1. Structures of test and other compounds.

replacements have no effect in the ivain series (cf. IX and XI). Conversely, a strong activity reduction was observed in the simultaneous oxidation of hydroxyl groups at C-2 and C-15 to the corresponding ketolactone (cf. XVI and XIX).

In agreement with observations reported by previous authors, the cleavage of epoxide ring at C-5 and of acetate groups at C-6 and C-18 causes the almost total disappearance of activity (cf. VI and VIII), although, as has been pointed out rightly by one of the referees, compounds in which only these acetate groups have been hydrolyzed, maintaining the epoxide moiety, have not been tested.

In short, from the above results we can conclude that the presence in the clerodane structure of one spiroepoxide substituent at C-4 and two acetate groups at C-6 and C-18, eventually, together with the hexahydrofurofuran moiety at C-9, is important to evoke activity, provided that no conformational distortion of the *trans* decalin system occurs by the action of other substituents, especially at C-1. In addition, a previously unreported enhancement of the activity by replacement of $\alpha$-hydrogen atom at C-2 by a hydroxy group has been observed. On the other hand, the poor antifeedant activities exhibited by simple butenolide

*Ajugareptansone A* VII

*Pentaol* VIII

*Ajugapitin* XIV

*14-Hydroajugapitin-2, 15-dione* XIX

XX

XXI R=R′= H
XXII R= Me; R′= H
XXIII R= Me; R′= $SO_2Ar$

FIG. 1. Continued.

derivatives XX–XXIII might suggest that this moiety would be not essential for eliciting antifeeding activity in ajugarins, although the study of more elaborate models is required to substantiate this hypothesis.

On the other hand, expression of antifeedant tests in terms of relationships between consumed food area and elapsed time can exhibit considerable variations, due to individual appetite differences among the test insects. The present evaluation procedure allows minimization of such deviation and, thus, it is suitable for comparison purposes. It is also worth pointing out that the time for reaching $CCD_{50}$ is not a significant parameter and may be different for every experiment.

Furthermore, other FRs corresponding to higher CCD values (i.e., $FR_{75}$) might be also eventually determined to estimate the persistency of phagorepellency or true absolute antifeedant activity (Hosozawa et al., 1974), when the choice test becomes progressively a nonchoice test. As shown in Table 1, comparison of results obtained with compound XVI at 0.1- and 1-$\mu$g doses illustrates the possible usefulness of other FRs at higher CCD measurements.

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# IDENTIFICATION AND BIOASSAY OF MACROCYCLIC LACTONE SEX PHEROMONE OF THE HALICTINE BEE *Lasioglossum zephyrum*

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**Abstract**—The macrocyclic lactones found in the Dufour's gland of the halictine bee *Lasioglossum zephyrum* are female sex pheromones. Octadecanolide, eicosanolide, docosanolide, and tetracosanolide, as well as monounsaturated homologs of each, are components of the Dufour's gland secretion of this species. Furthermore, a series of odd-carbon-numbered saturated and unsaturated hydrocarbons and isopentenyl docosanoate occur in extracts of the Dufour's gland and of whole females. Two different mixtures of all four synthetic lactones, and additionally a treatment consisting of the natural extract, elicit higher response levels than two of the lactones separately or either of the controls.

**Key Words**—*Lasioglossum zephyrum*, Halictidae, Hymenoptera, macrocyclic lactones, sex pheromones, kin recognition pheromones, Dufour's gland.

## INTRODUCTION

Macrocylic lactones have been identified from a wide variety of bees in the Halictinae and are used in the production of a protective lining for brood cells (Cane, 1981). In addition, lactones of similar chemical composition are used as defensive secretions in some termites (Prestwich, 1982) and might serve that purpose in halictine bees as well. However, the significance of macrocyclic lactones as sex pheromones in halictine bees has not been investigated to date. Our preliminary laboratory studies of male behavior in the primitively eusocial species *Lasioglossum zephyrum* (Hymenoptera: Halictidae) showed that the males

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are attracted to extracts of the Dufour's gland, which is connected to the female's sting and was formerly known as the basic gland (Lello, 1971). The primary chemical constituents of this gland in all species of the subfamily Halictinae so far studied are unbranched macrocyclic lactones with even carbon numbers between 16 and 26 inclusive (Cane, 1983; Bergström and Tengö, 1979; Hefetz et al., 1978; Johansson et al., 1982). The lactones may therefore play a major role in the sex pheromone communication system.

Furthermore, both males and females of *L. zephyrum* have the ability to distinguish between close and more distant kin (Greenberg, 1979; Smith, 1983). Males, after having been exposed to a female conspecific, are more highly attracted to other females not closely related genealogically to the first, and female guard bees allow female conspecifics to enter the nest if they are closely related to the guarding bee's nestmates. Such recognition has been shown, through behavioral experiments, to be mediated by a pheromone produced and emitted by the females (Bell, 1974; Barrows, 1975). The most parsimonious explanation would be that the same pheromone is used by males and females to distinguish among female kin.

The ability to recognize and thereby interact with close kin is a major postulate of theories explaining the evolution of social behavior (Hamilton, 1964), as well as theoretical explanations for nonrandom mating (Bateson, 1982). Since this bee species is so well studied with regard to the choices made by males during mating and by females at the nest entrances, it is appropriate to investigate the chemical communication system on which the behavior is based. A complex recognition mechanism is likely to be based on a mixture of several substances (Hölldobler and Michener, 1980), and the macrocyclic lactones produced by female *L. zephyrum* may prove to be major components of the kin recognition pheromone. This is a first report of the identification of some of the major chemical substances emitted by the females of *L. zephyrum*, as well as a biological assay of their potential as sex pheromones.

## METHODS & MATERIALS

*Chemical Identification.* Female bees extracted for chemical analysis were field collected throughout the summer from a nesting aggregation 25 km south of Lawrence, Kansas, and killed by freezing; most were probably workers with undeveloped ovaries. Workers in laboratory colonies remain very attractive to males, and therefore still produce the sex pheromone. The Dufour's gland was then dissected under saline solution by first removing the last four abdominal terga to expose the gland; the gland was then loosened from the other visceral material so that the unattached end floated free in the solution. A pair of fine forceps was then used to grip the gland as close to the base of the sting as possible and pull it free. After removal of excess water with a paper towel, the

gland was placed in a vial containing 300 μl of spectral grade, acid-washed, double-distilled hexane. Twenty glands were placed in each of eight vials containing hexane. Whole female extracts were prepared by placing 15 whole females in 300 μl of the same solvent.

Prior to chemical analysis, each extract was concentrated to approximately 50 μl by evaporation at 79°C. One glandular equivalent was then injected splitless onto an HP-5792 capillary gas chromatograph interfaced with an HP-3390 integrator. For quantification of the relative amounts of the lactones, coinjection with octacosane as an internal standard was made. Selected extracts were also analyzed on a Reibermag R 10/10 quadrupole GC-MS system interfaced with a PDP 11 computer system. Both machines were fitted with fused silica capillary columns coated with SE-30 stationary phase and programmed from 180 to either 210 or 220°C with an initial time of 5 min and a final isothermal time of 40 min. The gas chromatograph on the GC-MS system was equipped with a Ros injection system.

Following this initial analysis, extracts of female bees in hexane were purified on a preparatory GC equipped with a 2-m SE-30 packed column of 1/4 in. diameter. The eluted material was collected in four fractions as each fraction passed through thermal conductivity detector and was then subjected to further GC-MS analysis. The conditions for the GC-MS analysis were the same as above, except that the machine was fitted with a 25-m BP-20M fused silica capillary column and the final temperature was 220°C.

*Bioassay.* All biological assays were performed in the field during July 1983, on bright sunny days between 9 and 12 AM when the males were most active. The nest aggregation used for the tests was a large, nearly horizontal west-facing stream bank with several hundred active nests, the same one from which the bees were taken for extracts. All assays were based on the responses of males to various treatment substances.

The bioassay technique used is similar to that of Tengö (1979), in which a small square of black velvet is pinned approximately 1.5 cm above ground level in one of 10 previously chosen locations within the test area. Five microliters of each treatment were applied to the velvet immediately prior to the test and, prior to placement at the test location, 30 sec was allowed for evaporation of the hexane solvent (all solvent solutions were made such that 5 μl contained the amount to be tested).

In all cases the amounts used were based on molar equivalents and were relative to the total amount of lactones found in 0.5 female equivalents of the natural extract (Table 1). The treatments were as follows: control, hexane blank; odd odor, ethyl octadecadieneoate (not found in Dufour's gland extracts); natural extract (nat. ext.), 0.5 female equivalents from a hexane extract of whole, field-collected females; lactone mix 1, mixture of four synthetic lactones in the same ratio as in the natural extract; lactone mix 2, mixture of same four synthetic lactones in a different ratio than in the natural extract (Table 1); eicos-

TABLE 1. IDENTIFICATIONS FROM WHOLE-FEMALE *Lasioglossum zephyrum* EXTRACTS[a]

| Compound | % Total extract | Amt/Fem ($\times 10^{-6}$g) | MS-MW (% base) |
|---|---|---|---|
| 1 pentacosane | 15.5 | 3.4 | 352(38, M), 113(10), 99(12), 85(38), 71(71), 57(100) |
| 2 octadecenolide | 4.8 | 1.1 | 280(6, M), 260(3), 82(83), 73(8), 67(78), 60(4), 55(100) |
| 3 octadecanolide | 19.1 | 4.2 | 282(12, M), 264(9), 222(5), 83(50), 73(6), 69(69), 60(8), 55(100) |
| 4 eicosenolide | 2.0 | 0.5 | 308(9, M), 290(4), 248(1), 82(94), 73(8), 67(72), 55(100) |
| 5 eicosanolide | 21.2 | 4.7 | 310(16, M), 292(10), 250(4), 83(48), 73(13), 69(62), 60(8), 55(100) |
| 6 heptacosadiene[b] | — | trace | 376(30, M), 110(45), 96(75), 82(87), 67(76), 55(100) |
| 7 heptacosene[b] | — | trace | 378(40, M), 111(44), 97(70), 83(69), 69(76), 55(100) |
| 8 heptacosane[b] | — | trace | 380(12, M), 110(10), 99(11), 85(39), 71(67), 57(100) |
| 9 docosenolide | 11.0 | 2.4 | 336(10, M), 318(5), 276(1), 82(85), 73(6), 67(63), 60(3), 55(100) |
| 10 docosanolide | 16.1 | 3.6 | 338(22, M), 320(14), 278(4), 85(50), 73(13), 69(69), 60(7), 55(100) |
| 11 nonacosatriene[c] | — | trace | 402(40, m), 109(31), 96(53), 81(92), 67(97), 55(100) |
| 12 nonacosadiene (a) | 3.3 | 0.7 | 404(65, M), 109(30), 96(98), 82(94), 67(75), 55(100) |
| 13 nonacosadiene (b) | 0.6 | 0.1 | 404(82, M), 110(49), 96(62), 82(100), 67(63), 55(90) |
| 14 nonacosene | 1.5 | 0.4 | 406(46, M), 111(49), 97(80), 83(82), 69(85), 57(100) |
| 15 isopentenyl docosanoate | 1.7 | 0.4 | 408(0, M), 340(16), 324(14), 320(18), 85(18), 68(100), 60(2), 57(35) |
| 16 nonacosane | 0.7 | 0.2 | 408(9, M), 113(10), 99(12), 85(43), 71(68), 57(100) |
| 17 tetracosenolide[c] | — | — | 364(M), 346(3), 304(1), 82(100), 73(2), 67(56), 60(2), 55(89) |
| 18 tetracosanolide | 2.6 | 0.6 | 366(18, M), 348(12), 306(3), 83(48), 73(11), 69(66), 60(6), 55(100) |

[a]Listing of all the substances identified in extracts of undissected female *L. zephyrum*. Numbers to the left correspond to those in Figure 1A. Two isomers of nonacosene were resolved, corresponding

anolide, 20-carbon synthetic lactone only; and docosanolide, 22-carbon synthetic lactone only.

Ethyl octadecadieneoate was used as an "odd odor" because it has a chemical structure similar to that of the lactones (i.e., the ester group at one end, as well as a long hydrocarbon chain), although it elutes shortly before octadecanolide when coinjected with the female extract and is thus slightly more volatile. A series of ethyl esters of long-chain saturated and unsaturated fatty acids were components of some of the food (probably pollen) collected by workers. Thus, if the lactones are pheromones, they should be more attractive relative to the control than the "odd odor."

Each solution was tested five times per day over a four-day period. The sequence of the daily tests was randomized at the beginning of each test day, and the location for each test was randomly chosen. After placement of the treated black velvet square in the chosen location, the number of males approaching the square was counted (with the help of a hand counter) over the next 2 min. The criteria used for an approach are identical to the noncontact behaviors of Cane and Tengö (1981). In addition, the number of males making contact with the black velvet square after approaching was counted.

The number of approaches was first tested using an analysis of variance design (Sokal and Rohlf, 1982). However, the number of contacts could not meet the assumptions of ANOVA, so both the number of approaches and the number of contacts were also tested using the nonparametric procedures indicated in Table 2. Furthermore, in order to obtain the probability that a contact is made given that an approach occurs, the number of contacts was divided by the number of approaches for each individual test. After arcsin transformation, these data were tested using an ANOVA.

## RESULTS

The Dufour's gland extracts contained four of the lactones previously identified for halictine bees (Figure 1, Table 1), as well as monounsaturated homo-

to either *cis–trans* isomers or different positions of unsaturation. Tetracosenolide could not be resolved for accurate quantification and is, therefore, lumped with tetracosanolide for purposes of quantification. The amount for each compound was determined through the use of octacosane as an internal standard. The ratio among the lactones found in natural extracts is: 32.4% octadecanolide, 35.9% eicosanolide, 27.3% docosanolide, and 4.4% tetracosanolide (the monounsaturated lactones are considered together with the saturated lactones of the same carbon number). Thus, lactone mix 1 was made to match this ratio, and lactone mix 2 was as follows: 5% octadecanolide, 10% eicosanolide, 35% docosanolide, and 50% tetracosanolide. The total amount of lactone per female is 16.8 $\mu g$; therefore, the amount used for making up the solutions for the biological assays (0.5 female equivalents) was 8.4 $\mu g$.

[b]Compounds are present in all extracts, but not in sufficient amounts to accurately quantify.

[c]These compounds are not resolved from the ones listed immediately below them in the table; they were positively identified by peak scanning in the GC-MS analysis.

TABLE 2. RESULTS OF FIELD TESTS

| | Approaches | Contacts | Contacts/Approach |
|---|---|---|---|
| Control | 25.25(3.80) | 0.85(0.24) | 0.037(0.009) |
| Ethyl lineolate | 30.05(2.80) | 1.45(0.41) | 0.048(0.013) |
| Natural extract | 32.75(2.83) | 3.40(0.77)[b] | 0.096(0.019)[c] |
| Lactone mix 1 | 31.05(3.07) | 2.45(0.55)[b] | 0.082(0.018)[c] |
| Lactone mix 2 | 33.15(2.97) | 3.20(0.70)[b] | 0.088(0.018)[c] |
| Eicosanolide | 28.85(2.74) | 2.10(0.47) | 0.072(0.017) |
| Docosanolide | 31.30(3.78) | 1.70(0.54) | 0.039(0.011) |

[a] Means and standard errors of the means (parentheses) for the numbers of approaches, contacts, and contacts/approach for males of *Lasioglossum zephyrum* for each of the seven test groups. Samples size for each group was 20. The numbers of approaches were tested with both a Kruskal-Wallis test and ANOVA; no significant differences among the groups were apparent. The numbers of contacts were tested with a Kruskal-Wallis test only; the natural extract and the two mixtures of all four lactones were the only test groups significantly different from any of the other groups. The contacts/approach were tested with an ANOVA after arcsin transformation; the same three test groups were different from the control, ethyl linoleate and docosanolide. A posteriori tests were performed according to Sokal and Rohlf (1982).
[b] Different from control, $P < 0.05$.
[c] Different from control, ethyl linoleate, and docosanolide, $P < 0.05$.

logs of each. In addition to the lactones, the glands contained a series of hydrocarbons, all of odd carbon number, and isopentyl octadecanoate. The latter compound has been previously identified from Dufour's gland extracts of other North American halictine bees (Duffield et al., 1981).

Each of the four fractions obtained through preparatory gas chromatography corresponded to one of the four lactones and its monounsaturated homolog. In addition, two of the fractions contained the hydrocarbons identified in the initial GC-MS analysis. No new substances were found in the fractionated extracts. Furthermore, coinjection on the GC of each individual synthetic lactone together with the natural extract showed that the synthetic lactones corresponded in retention times to those of the analogous saturated natural lactones.

There are significant differences in the responses of males to the different treatments (Table 2). The treatments are not different in the number of approaches elicited, but both mixtures of all four saturated lactones, as well as the natural extract, elicited significantly higher numbers of contacts and contacts per approach than one or more of the other treatments. The probability of a contact after an approach was significantly higher in the two treatments containing all four synthetic lactones (mixtures 1 and 2) and the treatment made up of the natural extract than in the other treatments, which were not significantly different from the control. The three mixtures are also significantly more attractive than the odd odor and the docosanolide. The number of males varied

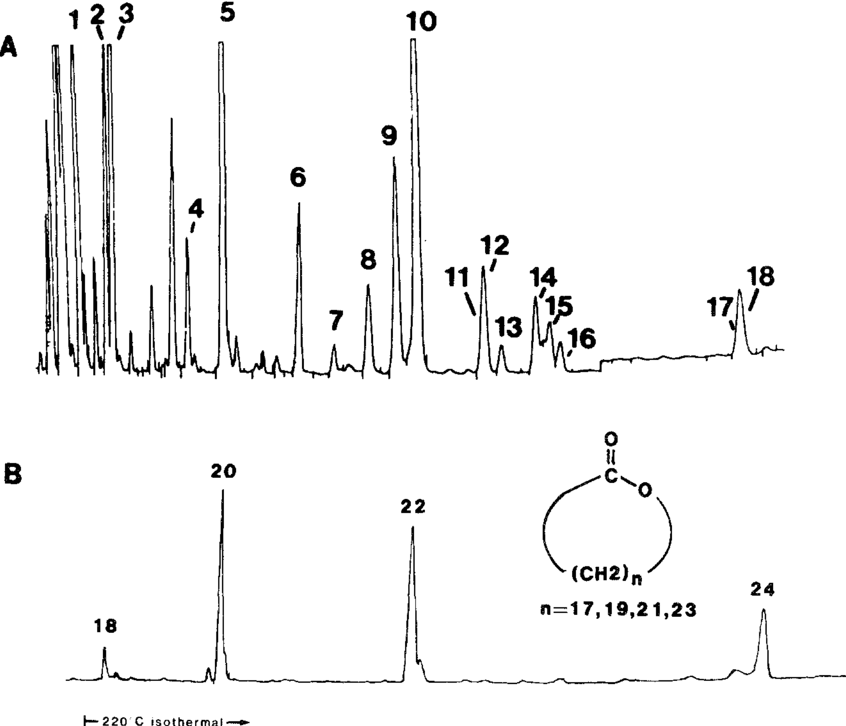


FIG. 1. Capillary gas chromatograph traces from extracts of undissected females of *L. zephyrum* (A) and the Dufour's gland extract (B). All of the hydrocarbons and the isopentenyl ester (Table 1) are present in very small quantities in the Dufour's gland extract as well and were identified by GC-MS analysis. The large, unnumbered peaks to the left in the upper chromatogram are ethyl esters of linolenic, linoleic, oleic, and stearic acids. These compounds were only intermittently present in the undissected female extract series, and we presently believe them to be extracted from the pollen or nectar collected by workers. Numbers in the top chromatogram correspond to the numbers in Table 1, and the numbers in the lower chromatogram indicate the numbers of carbons in each of the lactones. In neither case are tetracosanolide and tetracosenolide resolved from each other; however, GC-MS analysis with the more polar column (see text) and computer-directed peak scanning demonstrated the presence of both compounds in all extracts.

among the randomly chosen test areas; this variation tended to cloud the differences between the treatments. Moreover, males are attracted to odorless black spots (such as the black velvet squares), presumably visually (Barrows, 1975). Therefore, it is likely that factoring out the variation in number of approaches by analyzing the probability of contact per approach provides the best indication of the significant trends.

## DISCUSSION

The macrocyclic lactones found in the Dufour's gland of *L. zephyrum* play an important role in the recognition of females by males in this species; therefore, they cannot be ruled out as a possible source of the cues used in distinguishing between kin and nonkin. The occurrence of the different lactones in the same mixture increases the attractiveness of the mixture to the male bees, thereby demonstrating the potential importance of the pheromone mixture; the two individually presented lactones were each less attractive than mixtures of all four. Because macrocyclic lactones play a variety of roles in insects (Cane, 1981; Prestwich, 1982), it is not surprising that they are employed as sex pheromones and, possibly, for kin recognition in bees.

If, as indicated by the behavioral studies on *L. zephyrum* (Greenberg, 1979; Smith, 1983), there is significant genetic variation of alleles which code for the production of the pheromones, and if populations are differentiated with respect to these alleles, then significant differences in the amounts and/or ratios of the lactones might exist among populations of this species. Genetic and pheromone analyses show that nests of *L. zephyrum* are indeed differentiable with respect to the lactone ratios (Smith et al., in preparation; Crozier et al., in preparation). Furthermore, work with honey bees (*Apis mellifera*; Crewe, 1982) indicates that genetic variation specifies the ratios of the components in a pheromone mixture.

It is not surprising that female bees, when tested for attractiveness in the same nesting aggregation where the pheromones were tested (Smith and Wcislo, in preparation), were much more attractive to males than any of the synthetic pheromone mixtures. This finding indicates that there are dimensions of the communication channel between males and females of *L. zephyrum* which were not accounted for. Release rate of the lactone mixture may play an important role in the attraction of males. Extraction of whole females, or of whole Dufour's glands, gives a measure of the total amount of lactones contained per female; however, the amount released per unit time may not be a function of this total. Most of the pheromone substance is enclosed in the gland, and only smaller amounts may actually be exposed for release. Furthermore, visual effects were not accounted for in this experiment. Barrows (1975) reported that males, in the presence of the odor of a female, will "pounce" on black ink dots; presumably ink dots provide the same visual signal as the black velvet squares. But there may be some optimal size, color, and shape which represents the effective visual stimuli produced by a female.

The macrocyclic lactone mixture given off by females only mediates a part of the searching behavior of male *L. zephyrum*. Local search may be elicited by a combination of visual and olfactory cues and then followed by approach, which is culminated with either flying away or a copulation attempt. In our experiments the lactone mixtures did not elicit a higher number of approaches than

other odors, while it is clear that, since unextracted females elicited higher approaches than extracted ones (Smith and Wcislo, in preparation), some olfactory cue does mediate the approach stage. The combination of olfactory cues mediating the initial stage may be environmentally derived, such as food odors (odd odor), or other identified but untested compounds. Our data indicate, however, that once an approach is elicited, the lactones influence the probability that the approach will be culminated in a copulation attempt. Information on species identity and kinship is likely to be communicated over only a very short distance, and thus may very well be encoded in the lactone mixtures in *L. zephyrum*.

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## *Book Review*

**Chemical Ecology of Insects.** W.J. BELL AND R.T. CARDÉ (eds.). Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers, First published London, Chapman and Hall Ltd., 1984. $28.50(P), $45.00(C) ISBN 0-87893-069-8, ISBN 0-87893-070-1(pbk.). 524 pp.

The best short description of the present volume might be that it is six (or seven) books in one. The first being Perceptual Mechanisms (normally considered as physiology, but indispensable in this context). Is it mere chance that both authors, Städler (Contact Chemoreception) and Mustaparta (Olfaction) are Europeans, or does it reflect a difference in emphasis laid on biological approaches on both sides of the Atlantic? The editors Bell and Cardé, plus Elkington, continue with Odor Dispersion and Chemo-orientation Mechanisms. They first treat various dispersion models and consider walking and flying insects separately. These first five chapters cover roughly one quarter of the total text. Plant-Herbivore Relationships; Predators, Parasites, and Prey fill the next twenty percent of the book. Whether they cover one section or two (as is done by the editors) is a matter of taste. The first has two chapters, the second only one. Miller and Strickler (Finding and Accepting Host Plants) and Scriber (Host-Plant Suitability), whereas Vinson summarizes parasitoid–host relationships. The following five chapters cover, under two main headings (Chemical Protection and Chemical–Mediated Spacing) another quarter of the book. One of them, however, breaks through the chemical fence guarding the "ecology": it is Huheey's paper Warning Coloration and Mimicry. The chemical protection proper (by Nault & Phelan) is devoted to presocial insects. Resource Partitioning (Prokopy, Roitberg, and Averill); Aggregation in Bark Beetles (Birch), and Sexual Communication with Pheromones (Cardé and Baker) are brought together in the section on spacing. The remaining part of the volume is on sociochemicals of bees (Duffield, Wheeler, and Eickwort), ants (Bradshaw and Howse), and termites (Howse).

This enumeration of the total 16 chapters, each of a length between 10 and 37 pages already demonstrates that the editors have succeeded in bringing something new to the ever growing list of titles which, as a rule, contain only one or two of the six or seven topics mentioned above. One might argue, however, the validity of the term "ecology" in the title. The first sentence on the cover of the paperback edition gives a much more appropriate description: "a probe into the mechanisms involved in chemical signalling . . . " Chemical ecology is more



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than communication alone, and the book restricts itself in many instances not to that subdiscipline of biology. Apart from this, I consider the book as another attractive addition to the series of texts published by Sinauer during the last years. It brings together the discussions of specialists operating in their growing and consequently diverging fields, which share, however, many concepts and techniques. In my opinion, this makes the book especially useful for teachers and libraries rather than the student interested in one of the particular aspects only. It contains many references after each chapter; it is well illustrated; and the unavoidable printing errors are not abundant. A handsome book on chemical signaling.

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# HOST-PLANT PROTEIN AND PHENOLIC RESIN EFFECTS ON LARVAL GROWTH AND SURVIVAL OF A BUTTERFLY

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**Abstract**—*Euphydryas chalcedona* prediapause larvae were reared on fertilized and control shrubs of the host plant, *Diplacus aurantiacus.* Larval growth was enhanced by high leaf nitrogen content and inhibited by high leaf phenolic resin content. Larvae fed less on leaves near the branch tip which contained a higher leaf resin content. The results agree with prior laboratory investigation that the dietary content of nitrogen and *D. aurantiacus* leaf resin are major determinants of *E. chalcedona* larval growth and suggest that the phenolic leaf resin of *Diplacus* may both deter and inhibit leaf herbivores.

**Key Words**—*Diplacus aurantiacus*, Scrophulariaceae, *Euphydryas chalcedona*, Lepidoptera, Nymphalidae, flavonoid, herbivory, nitrogen.

## INTRODUCTION

Strong effects of leaf nitrogen content on insect herbivore feeding and nutrition have been demonstrated for a wide range of plants and insects (White, 1978; Mattson, 1980; Slansky and Feeny, 1977; Scriber and Slansky, 1981; Lincoln et al., 1982). Common responses to increased leaf nitrogen levels are increasing insect larval growth rates and decreased feeding rates with increasing available protein. Similarly, the effects of plant secondary chemicals on herbivores has also been widely demonstrated (Fraenkel, 1959; Ehrlich and Raven, 1964; Swain, 1977; Feeny, 1976). Many of the studies demonstrating the effects of secondary chemicals have taken place, however, under artificial diet or laboratory circumstances (Goldstein and Swain, 1965; Soo Hoo and Fraenkel, 1966; David and Gardner, 1966; Rice et al., 1978). Those studies which examined effects under more natural conditions have sometimes yielded results which con-



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tradicted the laboratory observations or suggested that leaf nitrogen content was a more important determinant of herbivore success than secondary chemical content (Fox and Macauley, 1977; Morrow and Fox, 1980; Bernays, 1981; Coley, 1983). Phenolic leaf chemicals, in particular, have been implicated in herbivore inhibition because of their hypothesized generalized mode of action of protein-complex formation (Goldstein and Swain, 1965; Feeny, 1970). Several studies have suggested that the proposed generalization mode of action may not occur and that phenols may not deter herbivores under field conditions (Fox and Macauley, 1977; Bernays, 1981; Zucker, 1983).

The objective of the present study was to test the effectiveness of *Diplacus aurantiacus* leaf resin in inhibition of herbivores. The growth and survival of *Euphydryas chalcedona* larvae were used to assess the leaf resin as well as the influence of leaf nitrogen under field conditions. *Diplacus aurantiacus* (Scrophulariaceae) produces large quantities (up to 30% of the leaf dry weight) of a low-molecular-weight phenolic resin on the external surfaces of the leaves (Lincoln, 1980). A previous laboratory study, using an artificial diet, has shown that the dietary content of *D. aurantiacus* resin chemicals reduces growth and survival of the larvae of the checkerspot butterfly, *Euphydryas chalcedona* (Lepidoptera: Nymphalidae), the principal herbivore of this plant (Lincoln et al., 1982). In addition, the influence of the resin interacted with the protein content of the food. High nitrogen content led to enhanced larval growth and survival, but the effect was dependent on the level of resin present in the diet. The effects of dietary nitrogen and resin were most striking at levels found in plants.

## METHODS AND MATERIALS

Ten pairs of plants were chosen at Jasper Ridge Biological Preserve, Stanford University, Stanford, California. Pairs of plants with similar vigor and size (1–1.5 m in height with multiple stems) were chosen. An area 1 m in radius was cleared around each plant and one pair member was fertilized on January 6, 1983 and March 29, 1983 with 10–10–10 slow-release NPK fertilizer to elevate its leaf protein content, while the other shrub remained as an untreated control. Inspection in May revealed that fertilized plants had grown much more than the control plants: fertilized plants had longer stems from the current year's growth than did unfertilized plants.

On May 23, 1983, 16 already-mated adult females of *E. chalcedona* were collected in a large subpopulation on Jasper Ridge where *D. aurantiacus* is the host plant. The adults were kept for two weeks, and egg masses were collected after oviposition on leafy stems of an alternative host plant, *Scrophularia californica.* Some egg masses were cooled to 6°C to synchronize hatching. On June 9, 1983, freshly hatched larvae were separated into batches of 50, and each group was placed on two *D. auranticus* leaves in a large vial.

After the larvae had spun a feeding web on the two leaves in the vial (two days), they were placed on shrubs. Each group of 50 larvae was attached to a leaf in the middle of a stem and a mesh bag slipped over the stem and sealed at the bottom. Each plant had two bags with 50 larvae in each. Bags were about 1 m in length and circumference and enclosed a sufficient amount of leaves to ensure that larvae would not lack for food and were situated so that the larvae could bask in the sun. The bags usually covered no more than 50% of the leaves of the plant. The mesh size of the bags were not small enough to retain the first-instar larvae, and probably not the second instar. The amount of light reduction due to the bags was approximately 5% (determined with a Lamda Instruments Quantum sensor which measures irradiance in the 400 to 700-nm band).

By June 17, 1983, larvae had spun visible feeding webs near the site where the larvae were originally placed. Although the number of larvae inside the bags could not be accurately estimated, no larvae were ever observed to have escaped from the bags despite repeated observations. The high survival at the end of the experiment ( >50%) confirms that there was little or no escape. On June 27, 1983, the primary branch of one bag broke at the base. The bag contained 24 larvae and, by this time, the larvae were too large to pass through the mesh bags. The data for this bag were retained and the larval weight at diapause was computed using the average larval weight in the other bag on the plant.

Rates of larval growth and development could not be measured because neither the number of active larvae nor the time of entry into diapause could be accurately determined. The experiment was therefore terminated when all larvae had obviously entered diapause (fourth instar). On July 13, 1983 all branches and intact bags were collected. The larvae were separated from the plants, counted, and weighed as a group. The average weight per larva was calculated by dividing the total larval weight by the number of larvae. Larval feeding was determined on 40 branches (20 from control plants and 20 from fertilized plants) by visually estimating the amount of leaf area consumed for the leaf pairs at the first six nodes below the branch tip. Feeding was estimated after all larvae had entered diapause. Branches which had not been fed upon at any nodal position were excluded.

Leafy branches were collected from each plant on June 11, 1983 and June 30, 1983 for chemical analyses of leaves and dried intact at 60°C. These dates correspond to the beginning of the experiment and an ending time when most of the larvae would have entered diapause. The nitrogen content was determined for a group of four to five leaves pooled from several positions on the branch for each plant on each date. Nitrogen content was determined with a Technicon Autoanalyzer II using Technicon Industrial Method 146171A and a modified digestion procedure of Isaac and Johnson (1976). From stems of each plant and date, leaf resin content was determined at three nodal positions, one to two expanded nodes below the tip, as well as two to four nodes and four to six nodes below the tip. Phenolic leaf resin content was determined by weighing individual

leaves, rinsing them with 5 ml of methanol, diluting 50-fold, measuring the absorbance at 292 nm, and assuming an average molecular weight of 440 and an extinction coefficient of $1.375 \times 10^4$ (Lincoln, 1980).

## RESULTS

The fertilizer treatment of the shrubs significantly increased the level of leaf nitrogen in the treated plants over the matched control plants by an average of 24% ($t = 5.20$, $P < 0.001$, $t$ test for paired comparison; Table 1). Leaf resin content, on the other hand, did not differ significantly between the two groups of plants at any of the three measured positions on the stem ($P > 0.05$, $t$ test for paired comparison), although the fertilized shrubs tended to have slightly higher levels than did the untreated controls.

Larval growth, measured by weight gain to diapause, differed significantly between treatment and control groups ($P < 0.046$, $t$ test for paired comparison; Table 1). Simple linear regression of the effect of leaf nitrogen on larval growth, however, did not reveal a significant effect. A general linear model approach was used for an analysis of covariance to assess the simultaneous effects of continuous variables (leaf nitrogen and resin contents) while adjusting for a class variable (treatment vs. control shrubs). Using a partial sum of squares (columns 3–5, Table 2), a statistically significant effect on larval growth was observed for the resin content of leaves at nodes 1–2 and nodes 4–6 below the tip, but not for leaf nitrogen content. A stepwise procedure was used to determine if the effects of resin were independent of possible effects of nitrogen. The stepwise method showed that leaf nitrogen had a very significant influence on larval growth (column 8, Table 2). The variance attributable to leaf nitrogen is also attributable to

TABLE 1. LARVAL PERFORMANCE AND HOST-PLANT CHARACTERISTICS FOR CONTROL AND FERTILIZED SHRUBS

| | Control | | Fertilized | |
|---|---|---|---|---|
| | Mean | Standard deviation | Mean | Standard deviation |
| Leaf nitrogen content (mg/g) (June 11 + June 30)/2 | 12.3 | 1.6 | 15.2 | 2.3 |
| Leaf resin content (mg/g) | | | | |
| Nodes 1–2 (June 11 + June 30)/2 | 233 | 42 | 256 | 17 |
| Nodes 2–4 (June 11 + June 30)/2 | 180 | 33 | 200 | 43 |
| Nodes 4–6 (June 11 + June 30)/2 | 136 | 31 | 160 | 31 |
| Survival (%) | 54.3 | 10.6 | 58.2 | 18.5 |
| Fresh weight per larva (mg) | 11.9 | 2.1 | 14.4 | 2.3 |
| Total larval biomass (mg) | 321.2 | 69.8 | 433.5 | 177.3 |

TABLE 2. ANALYSIS OF COVARIANCE FOR EFFECTS OF PLANT PAIR, LEAF NITROGEN, AND LEAF RESIN ON LARVAL GROWTH (FRESH WEIGHT AT DIAPAUSE), $R^2 = 0.88$

| | | Partial | | | Stepwise | | |
|---|---|---|---|---|---|---|---|
| Source | *df* | Sum of squares | *F* ratio | *P* < | Sum of squares | *F* ratio | *P* < |
| Pair | 9 | 76.8784 | 3.60 | 0.067 | 34.3879 | 1.61 | 0.289 |
| Leaf nitrogen | 1 | 8.5186 | 3.59 | 0.107 | 32.4021 | 13.65 | 0.010 |
| Leaf resin | | | | | | | |
| Nodes 1–2 | 1 | 30.9713 | 13.05 | 0.011 | 14.0083 | 5.90 | 0.051 |
| Nodes 2–4 | 1 | 10.1673 | 4.28 | 0.084 | 2.0913 | 0.88 | 0.384 |
| Nodes 4–6 | 1 | 24.5420 | 10.34 | 0.018 | 24.5420 | 10.34 | 0.018 |
| Error | 6 | 14.2392 | | | 14.2392 | | |

leaf resin at nodes 1–2 and nodes 2–4 (compare columns 5 and 8, Table 2), depending on the order of their entry into the computation. Thus, there appears to be a high degree of colinearity and lack of independence between the effect of leaf nitrogen and the effect of leaf resin at nodes 1–2 on larval growth. However, the effect of leaf resin at nodes 4–6 on larval growth was independent of leaf nitrogen.

Assessment of the influence of leaf resin on larval growth is best considered in the perspective of larval feeding. Larval feeding was reduced on leaves near the tip, especially for leaves at nodes 1–2 below the tip of the branch (Figure 1). This discrimination among leaves at different nodal positions did not differ appreciably between control and fertilized plants. The elongation of *D. aurantiacus* stems at Jasper Ridge is controlled by water availability and ceases in late June–early July (Mooney et al., 1980). Because of the limited potential for plant growth and the greater larval feeding in the latter stages of the experiment, the observed pattern of herbivory reflects larval feeding choice. The partial and stepwise regression coefficients suggested that the effect of leaf resin at nodes 1–2 on larval growth was positive, while the effect at nodes 4–6 was negative, with leaf resin at nodes 2–4 having little influence on larval growth. Hence, the influence of leaf resin is inhibitory (nodes 4–6) or potentially stimulatory (nodes 1–2). Because prediapause larvae of *E. chalcedona* feed primarily on leaves at nodes 3–6 below the tip (Figure 1), the positive correlation between larval growth and resin variation at nodes 1–2 appears to be spurious.

Larval survival did not differ significantly between the control and treated shrubs (Table 1). Survival was high, but was slightly lower on the control plants, perhaps because the initial attempt to place larvae on a control plant resulted in the lowest survival of any group (4%). In addition to a lack of difference between treatment and control, there was no statistically significant effect of leaf

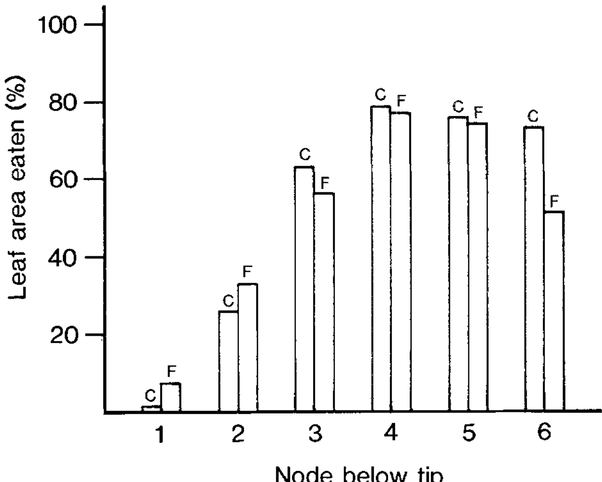


FIG. 1. Feeding of *Euphydryas chalcedona* prediapause larvae on control (C) and fertilized (F) shrubs of *Diplacus aurantiacus* according to leaf position below the tip of the branch (20 branches from each group).

nitrogen on survival and leaf resin content was significant only at nodes 1–2 (Table 3). As with the effect on larval growth, the regression coefficient of resin at nodes 1–2 on survival was positive, suggesting a stimulation of survival by leaf resin. There was, in addition, a significant difference in survival among plant pairs.

## DISCUSSION

The stimulatory effect of leaf nitrogen content on larval growth under field conditions is concordant with previous laboratory results for *Euphydryas chal-*

TABLE 3. ANALYSIS OF COVARIANCE FOR EFFECTS OF PLANT PAIR, LEAF NITROGEN, AND LEAF RESIN ON LARVAL SURVIVAL (PROPORTION SURVIVING AT DIAPAUSE), $R^2 = 0.92$

| | | Partial | | | Stepwise | | |
|---|---|---|---|---|---|---|---|
| Source | *df* | Sum of squares | *F* ratio | $P <$ | Sum of squares | *F* ratio | $P <$ |
| Pair | 9 | 0.2971 | 5.24 | 0.028 | 0.3333 | 5.88 | 0.022 |
| Leaf nitrogen | 1 | 0.0073 | 1.16 | 0.322 | 0.0057 | 0.91 | 0.377 |
| Leaf resin | | | | | | | |
| Nodes 1–2 | 1 | 0.0721 | 11.45 | 0.015 | 0.0661 | 10.50 | 0.018 |
| Nodes 2–4 | 1 | 0.0042 | 0.67 | 0.445 | 0.0004 | 0.06 | 0.812 |
| Nodes 4–6 | 1 | 0.0088 | 1.40 | 0.282 | 0.0088 | 1.40 | 0.282 |
| Error | 6 | 0.0378 | | | 0.0378 | | |

*cedona* prediapause larvae (Lincoln et al., 1982). In addition, the results also suggest that the growth of *E. chalcedona* is limited by leaf nitrogen content under field conditions. Leaf nitrogen level has long been hypothesized to be a limiting factor to the growth of individual insects and to insect population growth (Smith and Northcott, 1951; White, 1978; Mattson, 1980). The covariance of nitrogen content and resin yield at nodes 1–2 suggests that nitrogen fertilization led to a greater resin production. Some studies have suggested that phenolic chemical production was enhanced under conditions of low nitrogen supply (Janzen, 1974; McKey et al., 1978), and Mihaliak and Lincoln (1985) have shown that carbon allocation to volatile terpenes in the composite *Heterotheca subaxillaris* increases as nitrogen supply declines.

Phenolic leaf chemicals have been implicated in inhibition of specialist feeding herbivores because of their hypothesized generalized mode of action of protein-complex formation (Goldstein and Swain, 1965; Feeny, 1970). Several studies have found no evidence that the proposed generalized mode of action occurs and suggest that the presence and variation of leaf phenols may not be related to herbivore pressure (Fox and Macauley, 1977; Bernays, 1981; Coley, 1983; Zucker, 1983). The present evidence shows that for *E. chalcedona*, the level of leaf resin in *D. aurantiacus* leaves has a substantial influence on growth. Thus, these results confirm the reduction in larval growth by *Diplacus* resin observed in the previous study using controlled conditions and artificial diets. The principal resin constituents (flavonoids) contain an orthodihydroxy group (Lincoln, 1980), and the presence of this chemical group appears to enhance the lepidopteran larval growth inhibition of flavonoids (Elliger et al., 1980).

The limited effect of nitrogen or leaf resin on larval survival could be due simply to the greater degree of variability in survival compared to growth of individuals. It had been noted in the previous artificial diet study that measures of survival tend to be more variable than growth (Lincoln et al., 1982). A relatively high degree of variability among plants and even among pairs of bags on a single plant was observed in the present study. Alternatively, the significant difference in larval survival among plant pairs implies that factors beyond nitrogen or resin influence larval survival under field conditions. Presumably, large predators were not influential because of the protective net bags used in the experiment.

*Euphydryas* larvae have the capacity to both choose among leaves and to adjust feeding rate. Current and previous observations (Mooney et al., 1980) have shown that prediapause larvae of *Euhydryas chalcedona* feed on *Diplacus* leaves with low resin contents and on leaves several nodes below the branch tip (Williams, 1983). Larvae also chose leaves with low resin contents in the laboratory (unpublished data). Thus, the observed results suggest that the leaf resin acts as both a deterrent and an inhibitor of larval growth. Because older leaves at lower nodal positions have lower nitrogen levels (Mooney et al., 1981), inhibition of feeding near the tip of the stem could result in an inhibition of growth.

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# CONSUMPTION AND UTILIZATION OF EXPERIMENTALLY ALTERED CORN BY SOUTHERN ARMYWORM: Iron, Nitrogen, and Cyclic Hydroxamates

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**Abstract**—The effects of differential leaf water, leaf nitrogen and cyclic hydroxamate (DIMBOA) concentrations in corn seedlings were analyzed for a polyphagous insect, the southern armyworm (*Spodoptera eridania* Cram.). Six different combinations of nutrients and allelochemicals [DIMBOA = 2,4-dihydroxy-7-methoxy(2H)-benzoxazin-3(4H)-one] were generated using two corn genotypes (WF9 and CI3IA) and three fertility regimes (complete nutrient, Fe-deficient, and N-deficient solutions) in the University Biotron. Poorest larval growth was observed in the low-nitrogen treatments (1.2% and 1.7% leaf N) and was the result of both low consumption rates and high metabolic costs (low efficiency of conversion of digested food, ECD). Fastest growth rates were observed for the larvae fed leaves from the high-nitrogen treatments (4.6% and 4.4% leaf N). It is noteworthy that these treatments also contained the highest concentration of cyclic hydroxamates, which are generally believed to be the primary defensive chemicals mediating resistance against the European corn borer, *Ostrinia nubilalis* (Hubner). If these hydroxamates do have any deleterious or costly effects (perhaps accounting for a large portion of metabolic expenditures), the high digestibility of the leaf tissue and the increased consumption rates more than compensate, resulting in rapid growth (growth rate = consumption rate × approximate digestibility × efficiency of conversion of the digested food). These studies illustrate that variation in key nutrients and allelochemicals within a single plant species (*Zea mays* L.) may have significantly different effects upon various potential leaf-chewing caterpillars, such as these armyworms versus corn borers (which cannot handle the cyclic hydroxamates, even if provided with young nutritious leaf tissues).

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**Key Words**—Fertilization, *Zea mays*, iron deficiency, nitrogen deficiency, armyworm feeding, *Spodoptera eridania*, Lepidoptera, Noctuidae, DIMBOA, cyclic hydroxamates.

## INTRODUCTION

The role of cyclic hydroxamates in maize (corn) resistance to the European corn borer, *Ostrinia nubilalis* (Hubner), is one of the best studied phytochemical interactions between insects and a plant (Loomis et al., 1957; Klun et al., 1970; Scriber et al., 1975; Ortega et al., 1980; Guthrie, 1981). DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one) is the most abundant hydroxamic acid in maize extracts (Figure 1) (Corcuera et al, 1982, Argandona et al., 1982). Hydroxamic acids have been reported to play a role in plant resistance against several organisms (Virtanen, 1961; Corcuera et al., 1982). However, the specific mechanism of resistance to the corn borer remains unknown and appears to be

a. Glucoside I b. DIMBOA c. MBOA

d. Glucoside II e. DIBOA f. BOA

g. Glucoside III h. dimethoxy DIBOA i. dimethoxy BOA

FIG. 1 Primary glucosides in maize, *Zea mays*, and their breakdown products (after Manuwoto, 1980).

variable for different insects (see Manuwoto and Scriber, 1982; Scriber, 1984a). One of these insects of particular interest is the polyphagous southern armyworm, *Spodoptera eridania* Cram.

We were particularly interested in the responses of armyworms to differential phytochemistry as reflected by differential DIMBOA concentrations, nitrogen content, and leaf water content. We experimentally created six different natural phytochemical treatments by using different corn genotypes and fertility regimes.

Iron is an essential element for all aerobic organisms (Mengel and Kirby, 1982), and it plays a central part in life processes as a constituent of oxygen carrier molecules and as a versatile biocatalyst (Conrad et al., 1980). In plants, iron is crucial as a chloroplast constituent, being a component of ferredoxin (Possingham, 1971; Clarkson and Hanson, 1980). Green plants require a continuous supply of iron as they grow because iron is not translocated from the older to the newer leaves (Oertli and Jacobson, 1960; Brown, 1978). Since many microbes, in response to iron-deficiency stress, excrete chelating agents (most of these compounds are hydroxamic acids) which form stable complexes with $Fe^{3+}$ (Neilands, 1967, Raymond, 1977), we used a low-iron treatment to alter hydroxamate concentrations.

Nitrogen has been implicated as the major factor affecting herbivore survival, growth, and abundance (e.g., White, 1984). While nitrogen is doubtlessly a critical nutrient (see reviews in Mattson, 1980; Scriber, 1984b), its role relative to other leaf nutrients and allelochemicals and extrinsic environmental variables must be carefully considered (see Scriber and Slansky, 1981). We used a nitrogen-deficient nutrient solution for two corn genotypes with different hydroxamate production potential for this purpose.

## METHODS AND MATERIALS

Seeds of the corn genotypes C131A (a high DIMBOA genotype) and WF9 (a low DIMBOA genotype) were planted in moist silica sand in plastic dishpans (36.5 × 31 cm) at a density of 50 seeds per dishpan. Each corn genotype was subjected to three different nutrient solutions: (1) complete, (2) with an Fe deficiency, and (3) with a N deficiency (Table 1). The nutrient solution was a half-strength modified Hoagland solution (Schrader and Hageman, 1967). Each of three 200-liter plastic containers was filled with nutrient solution. Each nutrient container was connected to a pump which then was connected to a timer, so that corn plants were irrigated three times a day (at 6 AM, 12 noon, and 10 PM), each time for a 15-min period. These corn plants were fertilized and maintained in a controlled growth chamber in the University of Wisconsin Biotron with a 16 hr : 8 hr, light–dark photoperiod, a corresponding 30°C : 19°C temperature, and with a light intensity of 4400 ft-C.

TABLE 1. COMPOSITION OF NUTRIENTS USED TO IRRIGATE CI31A AND WF9 CORN GENOTYPES USED FOR FEEDING STUDIES

| Nutrient (mmol/liter)[a] | Corn nutrient treatment | | |
|---|---|---|---|
| | Complete | Fe-deficient | N-deficient |
| $MgSO_4$ | 2.00 | 2.00 | 2.00 |
| $KH_2PO_4$ | 0.50 | 0.50 | 0.50 |
| $K_2SO_4$ | 0.25 | 0.25 | 0.25 |
| $KNO_3$ | 2.50 | 2.50 | |
| $Ca(NO_3)_2$ | 2.50 | 2.50 | |
| $Mg(NO_3)_2$ | 2.50 | 2.50 | 0 |
| $Fe^{3+2b}$ | 0.30 | | 0.30 |
| $CaCl_2$ | 0.45 | 0.45 | 0.45 |
| Micronutrient[c] | | | |

[a] Half-strength Hoagland's solution.
[b] As chelate 330, Geigy Agricultural Chemical Co.
[c] As described by Schrader and Hageman (1967); Chevalier and Schrader (1977).

Corn seeds of each genotype and nutrient condition were planted at various times (three-day intervals) so that the corn plants fed to the larvae would be of comparable age over the period of feeding experiments. Fe and N deficiency symptoms appeared within 7–10 days, and approximately 20-day-old plants were fed to the southern armyworm larvae. Six phytochemical treatments (differential nitrogen, water, and DIMBOA concentrations) were generated by our genotype and fertility regimes (Table 2).

TABLE 2. LEAF WATER CONTENT, NITROGEN CONTENT, AND DIMBOA CONTENT OF TWO CORN GENOTYPES GROWN UNDER COMPLETE, FE-DEFICIENT, AND N-DEFICIENT NUTRIENT REGIMES[a]

| Treatment | $H_2O$ content | | N content | | DIMBOA | |
|---|---|---|---|---|---|---|
| | % fresh wt | *N* | % dry wt | *N* | mg/g dry wt | *N* |
| Fe-deficient (CI31A) | 92.1 ± 0.1 a | 26 | 4.4 ± 0.1 a | 6 | 1.7 ± 0.1 a | 4 |
| Complete (CI31A) | 90.9 ± 0.2 b | 21 | 4.6 ± 0.2 a | 6 | 1.3 ± 0.3 b | 4 |
| Fe-deficient (WF9) | 90.9 ± 0.1 b | 28 | 4.2 ± 0.0 b | 6 | 0.3 ± 0.1 d | 4 |
| Complete (WF9) | 90.0 ± 0.2 c | 28 | 3.7 ± 0.1 c | 6 | 0.2 ± 0.1 d | 4 |
| N-deficient (CI31A) | 87.8 ± 0.3 d | 21 | 1.7 ± 0.1 d | 6 | 0.5 ± 0.0 c | 4 |
| N-deficient (WF9) | 85.4 ± 0.3 e | 22 | 1.2 ± 0.1 e | 6 | 0.1 ± 0.0 d | 4 |

[a] All data were presented as mean ± SE. Means followed by the same letter in a column are not significantly different at $\alpha = 0.05$ by Duncan's multiple-range test.

Neonate larvae of the *S. eridania* were reared on baby lima bean plants, *Phaseolus lunatus* L. through the fourth instar. Fifteen freshly molted fifth-instar larvae were assigned to each of the six (corn genotypes/nutrient) treatments. The switch in host plants at this stage is normal in armyworm feeding ecology; nonetheless, within-instar effects (Scriber, 1981) and between-instar effects (Scriber, 1982) of host-plant switching upon southern armyworm can be significant. However, in the current study we are interested only in the relative differences between treatments. Feeding experiments were conducted on fifth- and sixth-instar larvae in a controlled growth chamber at 16 hr:8 hr/photo/scotophase with a corresponding thermoperiod of 23.5/19.5°C. Food consumption and utilization was estimated by a standard gravimetric technique (Waldbauer, 1968; Scriber, 1984a). Nutritional indices were calculated based upon the dry weight (biomass) of leaves, feces, and larvae. The mean larval weight during the stadium was estimated by (initial plus final weight)/2. Indices of larval performance are reported as in Scriber and Slansky (1981):

*GR*: growth rate (mg dry wt biomass gained per day) = $CR \times ECI$;
*RGR*: relative growth rate (mg biomass gained per day per mg larval biomass);
*CR*: consumption rate (mg dry wt food ingested per day);
*RCR*: relative consumption rate (mg biomass ingested per day per mg larval biomass).

AD, approximate digestibility (also called assimiliation efficiency)

$$= \frac{\text{food ingested (mg dry wt)} - \text{Feces (mg dry wt)}}{\text{Food ingested (mg dry wt)}} \times 100$$

ECD, efficiency of conversion of digested food (also called net growth efficiency)

$$= \frac{\text{Biomass gained (mg dry wt)}}{\text{Food ingested (mg dry wt)} - \text{Feces (mg dry wt)}} \times 100$$

ECI, efficiency of conversion of ingested food (also called gross growth efficiency)

$$= \frac{\text{Biomass gained (mg dry wt)}}{\text{Food ingested (mg dry wt)}} \times 100$$

$$ECI = AD \times ECD = \text{(overall efficiency)}$$

$$RGR = AD \times ECI \times RCR$$

Analyses of plant nitrogen and DIMBOA were done by a micro-Kjeldahl method (McKenzie and Wallace, 1954) and the ferric chloride method, respectively (Corcuera, 1974; Sullivan, 1975). We did not measure the actual concentrations of iron in the leaves in this study.

Analyses of variance were conducted on plant tissue quality ($H_2O$, N content and DIMBOA concentrations), and on performances of fifth- and sixth-instar larvae. Comparison of differences were obtained using Duncan's multiple range test (Snedecor and Cochran, 1967).

## RESULTS

Growth rate, consumption rate, and efficiency of penultimate (fifth) instar southern armyworm larvae were significantly suppressed in N-deficient treatments for both genotypes of corn (Table 3). Iron-deficient corn produced no significant suppression of growth rate except for sixth-instar larvae which were fed WF9 (Table 4). While leaves of the Fe-deficient treatments generally resulted in decreased digestibility and consumption rates, these were compensated for by increased conversion efficiencies of the digested food (ECD's; Tables 3 and 4). It can be seen in Table 5 and 6 that leaves of the Fe deficiency treatment reduced neither the total larval weight gained, prepupal weight, nor the instar duration. Higher DIMBOA concentration in Fe-deficient CI31A-corn did not seem to negatively affect the southern armyworm performances.

In contrast, N-deficient treatments appeared to have a severe effect upon the larvae. The lower RGR values are for the larvae fed N-deficient corn and slow growth is associated with both low consumption rates and low efficiencies (Table 3). In fact, none of the larvae survived the final (sixth) instar (Table 6) and only seven and three of the initial 15 larvae successfully completed the penultimate instar when fed upon N-deficient CI31A and WF9 corn (Table 3).

In this study, the growth rates of both penultimate and final instar larvae of *S. eridania* are significantly greater on CI31A leaves than on WF9 leaves (Figure 2). In a parallel experiment, we raised the southern armyworm larvae from first instar through pupation. The daily fresh weights of fifth- and sixth-instar larvae fed CI31A were always greater than those of larvae fed WF9 (Figure 3). The durations of fifth- and sixth-instar larvae fed CI31A were also shorter than those of larvae fed WF9. This is especially interesting in view of the deleterious effects of DIMBOA and the related cyclic hydroxamates in CI31A on the European corn borer, *Ostrinia nubilalis* Hubner, compared to the susceptible WF9 (Manuwoto, 1980; Guthrie, 1974, 1981).

## DISCUSSION

The results of this study should not be interpreted solely in terms of DIMBOA concentration, leaf water concentration, or nitrogen concentrations, although these nutrients are known to have a major impact upon the efficiency and rate of growth of many insects (see Figure 4 and reviews by Scriber, 1977;

TABLE 3. GROWTH RATE, CONSUMPTION RATE, AND EFFICIENCY OF FIFTH-INSTAR SOUTHERN ARMYWORM FED CI31A AND WF9 CORN GROWN UNDER COMPLETE, FE-DEFICIENT, AND N-DEFICIENT NUTRIENT REGIMES[a]

| | Growth rate | | Consumption rate | | Efficiency (%) | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | *AGR* (mg/day) | *RGR* (mg/mg/day) | *ACR* (mg/day) | *RCR* (mg/mg/day) | *AD* | *ECD* | *ECI* | Larvae (*N*) |
| FE-deficient (CI31A) | 5.0 ± 0.2 a | 0.48 ± 0.01 ab | 21.2 ± 2.6 a | 2.0 ± 0.2 ab | 60.2 ± 3.1 b | 46.5 ± 5.2 b | 27.2 ± 2.1 ab | 15 |
| Complete (CI31A) | 4.9 ± 0.4 a | 0.49 ± 0.02 a | 24.9 ± 2.2 a | 2.6 ± 0.3 a | 70.0 ± 2.8 a | 30.6 ± 3.4 bc | 22.1 ± 2.0 bc | 14 |
| Fe-deficient (WF9) | 3.7 ± 0.2 b | 0.41 ± 0.01 bc | 11.5 ± 0.8 bc | 1.3 ± 0.1 c | 53.1 ± 2.4 bc | 66.0 ± 5.3 a | 33.7 ± 1.8 a | 13 |
| Complete (WF9) | 3.4 ± 0.3 b | 0.37 ± 0.02 c | 18.7 ± 2.2 ab | 2.1 ± 0.2 ab | 68.4 ± 2.8 a | 28.0 ± 3.6 c | 22.4 ± 4.4 bc | 10 |
| N-deficient (CI31A) | 1.1 ± 0.3 c | 0.20 ± 0.03 d | 10.1 ± 1.2 c | 1.9 ± 0.2 bc | 60.5 ± 4.4 ab | 19.4 ± 3.9 cd | 14.3 ± 2.2 c | 7 |
| N-deficient (WF9) | 0.2 ± 0.1 d | 0.04 ± 0.01 e | 4.0 ± 1.2 c | 1.1 ± 0.4 c | 46.0 ± 6.6 c | 10.5 ± 5.9 d | 4.5 ± 1.8 a | 3 |

[a] All data were calculated as dry weight basis and presented as mean ± SE (*AGR* = absolute growth rate; *RGR* = relative growth rate; *ACR* = absolute consumption rate; *RCR* = relative consumption rate; *AD* = approximately digestibility, *ECD* = efficiency of conversion of digested food; *ECI* = efficiency of conversion of ingested food (Scriber and Slansky, 1981). Means followed by the same letter in a column are not significantly different at $\alpha = 0.05$ by Duncan's multiple-range test.

TABLE 4. GROWTH RATE, CONSUMPTION RATE, AND EFFICIENCY OF SIXTH-INSTAR SOUTHERN ARMYWORM FED CI31A AND WF9 CORN GROWN UNDER COMPLETE, FE-DEFICIENT, AND N-DEFICIENT NUTRIENT[a]

| Treatment | Growth rate | | Consumption rate | | Efficiency (%) | | | Larvae |
|---|---|---|---|---|---|---|---|---|
| | *AGR* (mg/day) | *RGR* (mg/mg/day) | *ACR* (mg/day) | *RCR* (mg/mg/day) | *AD* | *ECD* | *ECI* | (*N*) |
| Fe-deficient (CI31A) | 10.3 ± 0.6 a | 0.22 ± 0.01 a | 59.0 ± 3.0 b | 1.3 ± 0.0 c | 51.4 ± 1.7 b | 34.3 ± 1.8 a | 17.4 ± 0.5 a | 9 |
| Complete (CI31A) | 10.6 ± 0.3 a | 0.23 ± 0.01 a | 77.2 ± 3.8 a | 1.7 ± 0.1 ab | 67.4 ± 1.9 a | 20.8 ± 1.2 b | 13.9 ± 0.6 b | 9 |
| Fe-deficient (WF9) | 4.5 ± 0.6 c | 0.15 ± 0.01 c | 39.7 ± 3.8 c | 1.4 ± 0.1 bc | 56.6 ± 4.2 b | 22.6 ± 3.2 b | 11.8 ± 0.3 b | 9 |
| Complete (WF9) | 6.3 ± 0.9 b | 0.19 ± 0.02 b | 57.5 ± 7.9 b | 1.8 ± 0.2 a | 74.1 ± 2.7 a | 16.4 ± 2.2 b | 11.7 ± 1.2 b | 9 |
| N-deficient (CI31A)b | | | | | | | | |
| N-deficient (WF9)b | | | | | | | | |

[a] All data were calculated as dry weight basis and presented as mean ± SE. See Table 3 for explanation of abbreviations. Means followed by the same letter in a column are not significantly different at $\alpha = 0.05$ by Duncan's multiple-range test.
[b] All larvae died before completion of this instar.

TABLE 5. TOTAL FOOD CONSUMED, WEIGHT GAINED, AND DURATION OF PENULTIMATE INSTAR SOUTHERN ARMYWORM FED TWO CORN GENOTYPES GROWN UNDER COMPLETE, FE-DEFICIENT, AND N-DEFICIENT NUTRIENT REGIMES[a]

| Treatment | Total food consumed (mg) | Larval weight gained (mg) | Duration (days) | N |
|---|---|---|---|---|
| Fe-deficient (CI31A) | 59.1 ± 6.7 a | 14.0 ± 0.4 a | 2.8 ± 0.1 c | 15 |
| Complete (CI31A) | 58.8 ± 5.3 a | 11.4 ± 0.4 b | 2.4 ± 0.1 c | 14 |
| Fe-deficient (WF9) | 32.7 ± 2.4 bc | 10.6 ± 0.3 bc | 2.9 ± 0.5 c | 13 |
| Complete (WF9) | 52.6 ± 6.4 ab | 9.3 ± 0.6 c | 2.8 ± 0.1 c | 10 |
| N-deficient (CI31A) | 41.2 ± 3.5 abc | 4.6 ± 0.8 d | 4.3 ± 0.4 b | 7 |
| N-deficent (WF9) | 26.9 ± 7.7 c | 1.1 ± 0.1 e | 7.0 ± 0.5 a | 3 |

[a] Total food consumed and larval weight gained were calculated as dry weight basis and presented as mean ± SE. Means followed by the same letter in a column are not significantly different at $\alpha = 0.05$ by Duncan's multiple-range test.

Mattson, 1980; Scriber and Slansky, 1981; Slansky and Scriber, 1985). Plants deficient in minerals are also unlikely to be normal physiologically and may contain atypical concentrations of organic or inorganic compounds that can affect the growth of insects feeding on them (Friend, 1958; see also Reese 1979, Scriber and Mattson, 1985). Due to a depression of the activity of aconitase, iron-deficient corn may, for example, contain more citric acid and less malic acid than normal corn leaves. Furthermore they may contain a higher K:Ca ratio than normal leaves (Palmer et al., 1963). Bar-Akiva (1971) reported that

TABLE 6. TOTAL FOOD CONSUMED, LARVAL WEIGHT GAINED, PREPUPAL WEIGHT, AND DURATION OF FIELD INSTAR SOUTHERN ARMYWORM FED CI31A AND WF9 CORN GROWN UNDER COMPLETE, FE-DEFICIENT, AND N-DEFICIENT NUTRIENT REGIMES[a]

| Treatment | Total food consumed (mg) | Larval weight gained (mg) | Prepupal weight (mg) | Duration (days) | N |
|---|---|---|---|---|---|
| Fe-deficient (CI31A) | 346.0 ± 23.1 bc | 59.9 ± 3.8 a | 76.6 ± 4.0 a | 5.9 ± 0.2 b | 9 |
| Complete (CI31A) | 468.6 ± 30.3 a | 57.1 ± 6.6 a | 77.3 ± 2.7 a | 6.0 ± 0.1 ab | 9 |
| Fe-deficient (WF9) | 271.4 ± 22.0 c | 31.6 ± 2.9 b | 44.7 ± 2.7 b | 7.0 ± 0.2 a | 9 |
| Complete (WF9) | 372.1 ± 37.6 b | 41.1 ± 3.6 b | 52.5 ± 4.2 b | 7.0 ± 0.6 a | 9 |
| N-deficient (CI31A)b | | | | | 0 |
| N-deficient (WF9)b | | | | | 0 |

[a] Total food consumed, larval weight gained, and prepupal weight were calculated as dry weight basis and presented as mean ± SE. Means followed by the same letter in a column are not significantly different at $\alpha = 0.05$ by Duncan's multiple-range test.
[b] All larvae died before completion of this instar.

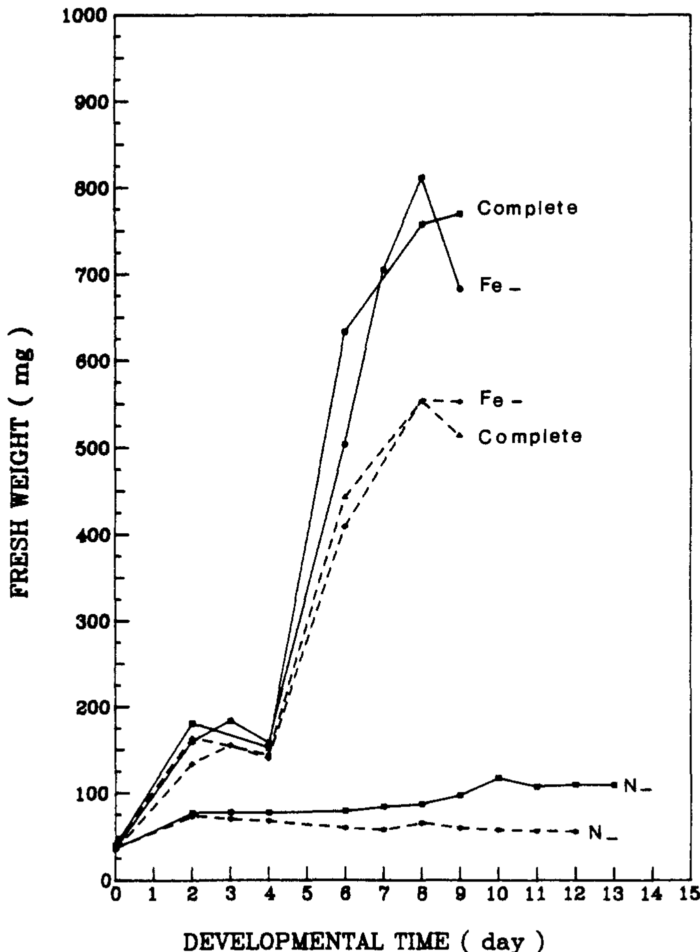


FIG. 2. The effect of complete, Fe-deficient, and N-deficient CI31A and WF9 on fresh larval weight of *S. eridania* during fifth- and sixth-instar development (solid lines = CI31A, dashed lines = WF9).

Fe deficiency caused the accumulation of asparagine in citrus, corn, and rye. Thus, there are many factors other than $H_2O$ and N content and DIMBOA concentration (see also Corcuera et al., 1982) which may be involved in determining the southern armyworm responses in this study. This study showed that corn Fe-deficient regimes resulted in significantly decreased consumption rates and digestibilities of the southern armyworm larvae, even though the substantial effect on growth rate was not observed. This study also demonstrated that leaves with 1-2% N content did not support a normal growth of the southern armyworm larvae.

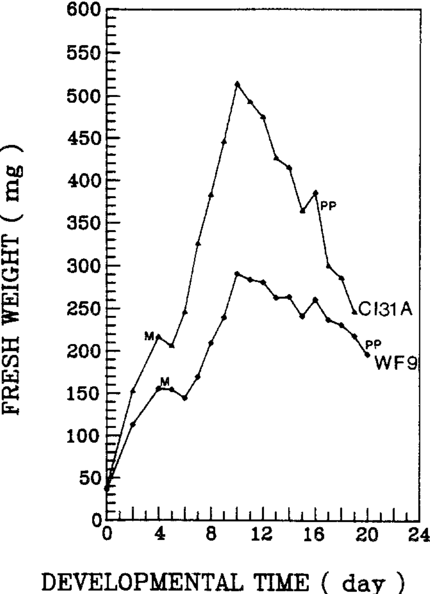


FIG. 3. The growth curve of fifth- and sixth-instar larvae of *S. eridania* reared on CI31A and WF9 from neonate larvae. (M = molting, PP = prepupal).

There is little information regarding the direct response of phytophagous insects to bioelements in their food plants (Larsson and Tenow, 1979) and even less regarding elements such as iron supplied to the food plant. Allen and Selman (1955) reported a significant decrease in the oviposition rate of the mustard beetle (*Phaedon cochlearia*) fed on watercress leaves deficient in Fe. For many insects, iron is one of several metals which is a dietary essential because of functions as cofactors in enzymatic reactions (Dadd, 1973). Mattson (1983) reports that spruce budworm (*Choristoneura fumiferana*) growth is positively linked to foliar N but negatively linked to Fe and K. He also reports that iron levels in the insect body show a tendency to decrease with increased iron levels in the diet. Furthermore iron concentrations in the budworms are negatively correlated with insect size, and Fe is the only element showing this inverse behavior (Mattson, 1983). However, the range of iron requirements are not yet known for insects in general, nor are the Fe dynamics in plants under various environmental and biological stresses (Scriber and Mattson, 1985).

## CONCLUSIONS

Armyworms are affected by the nutritional and/or allelochemical changes in their food plants. In our study with two maize genotypes, the different fertility

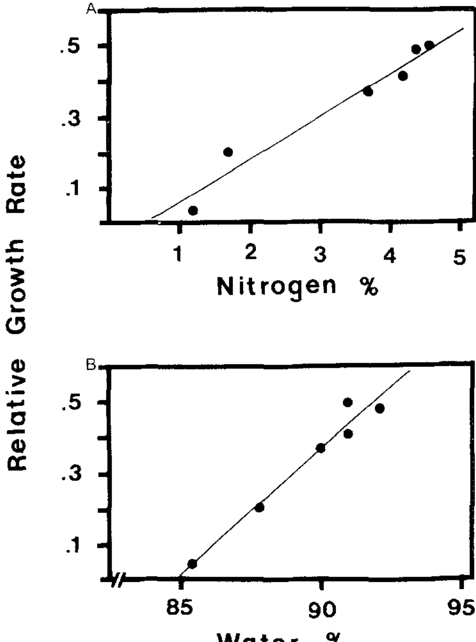


FIG. 4. (A) Growth rate as a function of corn leaf nitrogen (% dry weight). ($Y = 0.05X + 0.12$; $r = 0.979$). (B) Growth rate as a function of corn leaf water content (% fresh weight). ($Y = -15.97X + 0.07$; $r = 0.983$).

regimes (complete, Fe-deficient, and N-deficient) significantly affected leaf water and nitrogen content as well as the concentration of DIMBOA in the leaf tissues. These changes differentially affected the consumption rate, digestion, and conversion of plant biomass by the southern armyworm.

Perhaps the most intriguing result from this study is that insect growth was significantly faster for all instars of the southern armyworm on the high-DIMBOA treatments than on the low-DIMBOA (European corn borer-"susceptible") treatments. The implications of these results are that greater consumption rates and faster growth rates may result for certain insects on plant genotypes bred specifically for high allelochemic concentrations (e.g., DIMBOA) against other pathogens or insects (see also Scriber, 1979, 1984b).

Thus, in the development of comprehensive pest management programs through plant breeding, we must consider not only the target pest, but all possible pests. In addition, we need more information on the impact of different fertility regimes upon phytochemical dynamics and the subsequent consequences of these changes upon the herbivorous insect populations. A recent review (Scriber, 1984b) addresses our current state of ignorance in this area.

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# POLYUNSATURATED HYDROCARBONS IN THE STABLE FLY[1]

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**Abstract**—Three triply-unsaturated hydrocarbons were identified from cuticular lipids of male and mixed-sex stable flies, *Stomoxys calcitrans*. The major compound, (Z,Z)-1,7,13-pentacosatriene, and two minor compounds, (Z,Z)-1,7,13-tetracosatriene and (Z,Z)-1,7,13-tricosatriene, were synthesized. Samples of male and female stable flies that differed in age, seasonality, geographic origin, rearing conditions of adults, and methods of extraction were analyzed for the presence of these triolefins. Females were found to have small quantities of the same $C_{25}$ triolefin, which appeared to be identical to that in males. No evidence was seen for attraction of males or females to natural or synthetic triolefins.

**Key Words**—Stable fly, *Stomoxys calcitrans*, Diptera, Muscidae, hydrocarbons, olefins, (Z,Z)-1,7,13-pentacosatriene, (Z,Z)-1,7,13-tetracosatriene, (Z,Z)-1,7,13-tricosatriene.

## INTRODUCTION

Muhammed et al. (1975) reported the presence of a sex attractant pheromone for males in whole-body extracts of the female stable fly, *Stomoxys calcitrans* L. The active materials appeared to be polyunsaturated hydrocarbons present in modest quantities in mature females. In tests with two- or four-port olfactometers, male flies responded to an airstream that passed downward over treated filter paper, then through screen cones, and into a chamber which held 20 (two-port olfactometer) or 40 males (four-port olfactometer). Flies that had moved onto or through the choice ports in 15 min were counted. Most activity was

[1]Mention of a proprietary or commercial product does not constitute an endorsement by either the U.S. Department of Agriculture or the University of Florida.

obtained in the hexane–ether (99 : 1) eluate from silica gel liquid chromatography (LC), when 23% of the mature males were attracted with 10 female equivalents, compared to 6% to the blank. Three polyolefins collected from this fraction by preparative gas chromatography (GC) had 23, 24, and 25 carbons and were attractive in olfactometer tests after GC determination of purity and quantitation. Males contained about four times more total polyolefin than females, including the major $C_{25}$ components; no further identification work was reported (Muhammed, 1975).

In a companion report, Uebel et al. (1975), reported this class of compounds in male flies, but not in females isolated since eclosion. These workers identified and synthesized one component from male flies, (*Z,Z*)-1,7,13-pentacosatriene (Sonnet et al., 1977), and its geometric isomers were synthesized (Sonnet, 1979).

Owing to conflicting reports concerning the presence and quantity of $C_{25}$ polyolefin in female flies, we reinvestigated the origin of this compound in male and female flies and its biological activity. The identification and synthesis of the 23-, 24-, and 25-carbon compounds found in stable fly polyolefins are described. The parameters considered in these tests were geographic location, seasonality, rearing conditions of adults, and methods of extraction.

## METHODS AND MATERIALS

*Biological Material.* Flies used in this study were originally obtained from flies colonized at the Gainesville laboratory in 1976, 1977, and 1984 or from pupae collected in the wild at St. Croix, U.S. Virgin Islands in 1976. All adult flies were reared in cages as previously described (Muhammed et al., 1975).

The $C_{25}$ polyolefin content of "summer" (reared in June 1976) and "winter" (reared in January 1977) flies of the Gainesville laboratory strain and the wild St. Croix flies (reared in March 1977) was determined.

In one series of tests, 500–1000 newly eclosed flies were sexed within 24 hr of emergence, and maintained under various conditions described below before extraction: (1) 24 hr: flies immediately frozen; (2) virgin: flies sexed within 24 hr of emergence, then maintained for 5 days with consexuals; (3) semiisolated: Females sealed in a small paper tub with the bottom and portions of the sides removed and covered with three layers of tube gauze and the tub placed within a larger cage containing an equal number of males for 5 days; (4) pupae: isolated in individual vials until eclosion, and the adults reared with consexuals for 5 days; (5) mixed sexes (mated): newly emerged flies were held together for 5 days, then sexed. Females in (1) through (4) were sexed again before extraction.

All bioassays were conducted using equipment and techniques as described by Muhammed et al. (1975).

*Extraction.* Crude extracts of the variously reared flies were made in two separate steps in the following manner: (1) Frozen flies were immersed in 50 ml hexane for 24 hr, and the solvent decanted through filter paper; this procedure was repeated once more, and the extracts combined as the initial cuticular wash. (2) The same flies were immersed in 50 ml hexane for 5 days before decanting; flies were then ground in a mortar and reimmersed in hexane overnight before decanting. Both extracts were combined as the final wash.

Spermathecae from 12 mature males (5–6 days old) were dissected in water, transferred to a glass vial, dried, and extracted with hexane for GC.

*Isolation and Analysis.* Extracts were fractionated on 2 × 45-cm columns of silica gel (60–200 mesh, Baker); saturated and unsaturated hydrocarbons eluted with 200 ml of hexane, polyunsaturated hydrocarbons with 200 ml of 1% ether in hexane, and other lipids with 100 ml each of 10 and 50% ether in hexane. The hydrocarbons were separated into paraffins and olefins on 2 × 45-cm columns packed with 20% silver nitrate impregnated silica gel (60–200 mesh, HI-FLOSIL-AG, Analabs). The purity of each of these classes of compounds, including the polyunsaturates from all rearing regimens, was checked by thin-layer chromatography (TLC) on silica gel plates impregnated with 20% silver nitrate (250-μm Uniplates, Analtech) and compared with paraffin and olefin standards. Quantification was done by temperature-programed GC on a Varian 2100 with glass columns (1.8 m × 2 mm ID) of 3% SE-30 or a Varian 3700 with a fused silica DB-1 column (15 m × 0.23 mm) (Figure 1, Table 1). Preparative GC samples were separated on an aluminum column (3 m × 4 mm ID) packed with 5% SE-30 in a Varian 1400 using a splitter.

Spectra were obtained on a Perkin Elmer model 221G infrared spectophotometer in solutions of $CCl_4$ and as films to determine double-bond configuration of compounds from polyolefin fractions and synthesized materials.

Sites of unsaturation of three polyolefins were determined by microozonolysis (of samples separated by preparative GC) and GC analysis of the resulting aldehyde and dialdehyde fragments after the method of Beroza and Bierl (1967). Natural III was hydrogenated over neutral palladium catalyst to confirm the absence of branching by electron impact mass spectra (EI-MS) using a Varian MAT CH5 mass spectrometer (GC-MS) via a 3.2 m × 2 mm ID glass column of 3% OV-1. Methane chemical ionization mass spectra (CI-MS) were obtained to determine the molecular weights of I, II, and III and the aldehyde ozonolysis fragments of III with a Finnigan 1015C GC-MS having 1.8 m × 2 mm stainless-steel columns of 5% SE-30 or 5% HI-EFF-1-BP. Methane CI-MS analyses were repeated in 1984 with a Finnigan 4000 EI/CI GC-MS system via a fused silica capillary (DB-1, 15 m × 0.23 mm).

*Synthesis.* The major triene III was synthesized by the alkylation of 1 mol of 1,7,13-tetradecatriyne (Farchan) with 1 mol of 1-bromoundecane, using *n*-butyllithium and THF–HEMPA as solvents (Figure 2). Silica gel chromatogra-

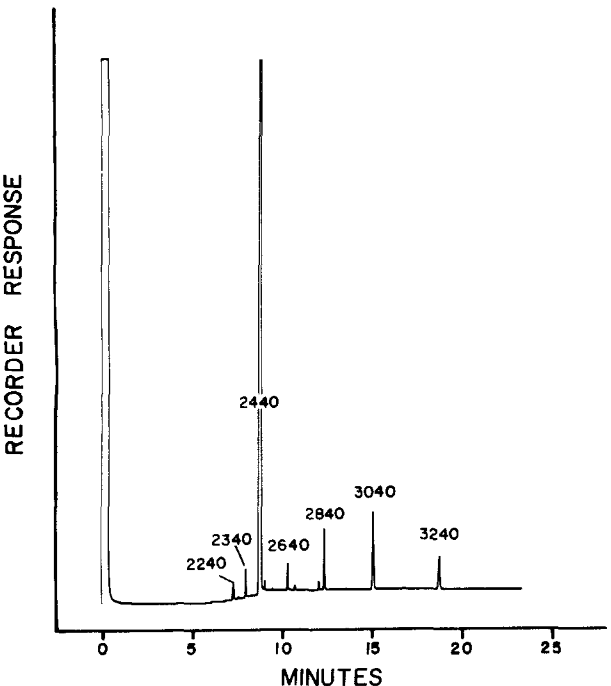


FIG. 1. Temperature programmed (200–300° at 12°/min) gas chromatogram of polyolefins from 1000 mixed sex stable flies on a fused silica DB-1 column (15 m × 0.23 mm ID), $H_2$ carrier gas.

phy of the monoalkylation product gave 60% yield of $C_{25}$ triyne. Hydrogenation at 1 atm over 5% palladium catalyst poisoned with quinoline was followed by GC until the peak for the triene (KI2440) was maximized at 4 hr. The product, analyzed on silver nitrate TLC plates developed with 20% benzene in hexane, showed small amounts of *trans* compounds at $R_f$ 0.7. The majority cochromatographed with the natural product, which also showed a smear at $R_f$ 0.3–0.6. Silver nitrate chromatography on a 1 × 45-cm column gave a 60% yield of III from the triyne in the third fraction of 50% ether in hexane (100 ml), after hexane (150 ml) and 10% ether in hexane (100 ml) fractions has ben collected.

The mass spectrum of synthesized III was identical to that of natural III. Ozonolysis gave essentially the same aldehydes and dialdehydes by GC as natural III. A sample of III independently synthesized (Sonnet et al., 1977) was coincident by GC-MS and ozonolysis.

Homologs were prepared by addition of either 1-bromononane or 1-bromodecane to 1,7,13-tetradecatriyne followed by hydrogenation of the products as described to give (*Z,Z*)-1,7,13-tricosatriene (I) and (*Z,Z*)-1,7,13-tetracosa-

TABLE 1. PENTACOSATRIENE (III) FOUND IN EXTRACTS OF VARIOUSLY REARED 5-DAY-OLD STABLE FLIES[a]

| Condition of flies[b] | No. flies | Micrograms found per fly | |
|---|---|---|---|
| | | Initial wash[b] | Total[c] |
| Females | | | |
| 24 hr | 1028 | 0.015 | |
| Virgin | 668 | 0.085 (81%) | 0.105 |
| Semiisolated | 553 | 0.048 | |
| Isolated | 309 | 0.007 (70%) | 0.010 |
| Mated | 511 | 3.82 | |
| Males | | | |
| 24 hr | 1094 | 0.020 | |
| Virgin | 539 | 8.30 (68%) | 12.27 |
| Semiisolated | 449 | 10.96 | |
| Isolated | 254 | 2.46 (53%) | 4.64 |
| Mated | 437 | 7.25 | |

[a] Determined by GC on 1.8 m × 2 mm ID glass column of 5% SE-30 on Gas Chrom Q (120–140 mesh).
[b] Refer to text for details.
[c] Combined extracts of the "initial wash" and "final wash" as described in the text.

triene (II). These compounds were separated on silver nitrate chromatography and were identical by ozonolysis, GC and CI-MS to natural I and II.

## RESULTS

*Identification.* Retention indices for natural polyolefins were KI2240 (I), KI2340 (II), KI2440 (III), KI2640, KI2840, KI3040, and KI3240 (Kovats, 1965, Figure 1).

Infrared spectra showed vinyl (987, 902 $cm^{-1}$) and *cis* (739 $cm^{-1}$) adsorptions for the polyolefin fraction and for III after separation by preparative GC from I and II. The spectra obtained by methane CI-MS showed a small M strad-

$$HC{\equiv}C(CH_2)_4C{\equiv}C(CH_2)_4C{\equiv}CH + CH_3(CH_2)_{10}Br \xrightarrow[\text{HEMPA}]{\text{nBuLi, THF}}$$

$$HC{\equiv}C(CH_2)_4C{\equiv}C(CH_2)_4C{\equiv}C\text{-}(CH_2)_{10}CH_3 \xrightarrow[\text{Pd/BaSO}_4\ \text{Quinoline}]{H_2}$$

$$HC{=}C(CH_2)_4C{=}C(CH_2)_4C{=}C(CH_2)_{10}CH_3$$

FIG. 2. Synthesis of 1,7,13-pentacosatriene (III).

dled by larger M − 1 and M + 1, characteristic for unsaturated hydrocarbons: I (*m/z* 317, 318, 319), II (*m/z* 331, 332, 333), and III (*m/z* 345, 346, 347). Thus, the molecular weights were 318, 332, and 346, respectively, with three double bonds in each.

Natural III was hydrogenated to a paraffin that eluted with *n*-pentacosane (KI2500) on an SE-30 column and showed a molecular ion at *m/z* 352 by EI-MS coincident with that of *n*-pentacosane.

Compounds that eluted later than III by GC, apparently in the same chemical class (triolefins), did not give visible parent ions with methane ionizing gas in early studies, and reconstructed ion chromatograms were not helpful. Later spectra obtained using isobutane CI-MS showed the presence of two $C_{25}$ dienes (*m/z* 348) at KI2475 and 2465, and what was apparently a homologous series of diolefins including a $C_{27}$ (*m/z* 374) at KI2655 and a $C_{29}$ (*m/z* 402) at KI2855. These were present in an incompletely separated $AgNO_3$ column eluate of the triolefin fraction at about the same level as I and II (1–2%). Capillary CI/GC-MS of 1984 samples recovered from the lower half of the $AgNO_3$ TLC spot showed a clean pattern of homologous triolefins that appeared at KI2640 (0.2%, CI-MS mol. ion at *m/z* 374), 2840 (0.5%, *m/z* 402), 3040 (1.7%, *m/z* 430), and 3240 (0.5%, *m/z* 458) in 1984 mixed-sex flies.

These triolefins, quantified for each sex in 1984 (Table 2) were present in 1976 and 1983 colony flies in about the same quantities. In addition to III, recently studied (1984) males had more I and II than females, whereas mated females had ca. 5× more of the higher homologs that were also present in virgin females. Body parts from 1984 flies of both sexes were analyzed for III (Table 3), using the total hydrocarbon fraction, as III was well separated from other materials. Males and mated females had similar distribution of III, with the largest proportion on legs, and again the males had nearly 5 times more. In unmated females sexed within 18 hr, only abdomens contained III, and other

TABLE 2. POLYOLEFINS IN EXTRACTS OF STABLE FLIES ($\mu$g/fly)[a]

| KI | Males (m, 4–5 days)[b] | females (m, 4–5 days) |
|---|---|---|
| 2240 | 0.002 | nd |
| 2340 | 0.005 | 0.014 |
| 2440 | 11.19 | 0.048 |
| 2640 | 0.015 | 0.073 |
| 2840 | 0.010 | 0.083 |
| 3040 | 0.015 | 0.071 |
| 3240 | 0.010 | 0.046 |

[a] $AgNO_3$ TLC plate scrapes, 1984 flies; m = mated.
[b] From male abdomen sample × 5.328.

TABLE 3. PENTACOSATRIENE (III) IN-POOLED HYDROCARBONS FROM BLOOD-FED STABLE FLY BODY PARTS (μg/fly)

| | Heads | Thoraces | Abdomens | Legs | Wings | Total |
|---|---|---|---|---|---|---|
| Females | | | | | | |
| Unmated (3 days)[a] | 0.0006 | 0.029 | 0.502 | 0.005 | 0.0015 | 0.54 |
| Mated (4–5 days)[b] | 0.032 | 0.510 | 0.529 | 1.192 | 0.320 | 2.58 |
| Isolated (3 days)[c] | ND | ND | ND | ND | ND | |
| Males | | | | | | |
| Mated (4–5 days)[b] | 0.23 | 1.90 | 2.67 | 3.54 | 2.85 | 11.19 |

[a] Flies sexed within 18 hr of emergence ($N = 30$).
[b] $N = 30$.
[c] Virgin females emerged and reared in individual vials ($N = 8$).

body parts had only a trace. In contrast, III was not detected in a small sample ($N = 8$) of individually reared, isolated females, which agrees with Table 4.

Ozonolysis of the separated three major triolefins indicated that they formed a homologous series, as major cleavage fragments were decanal, undecanal, and dodecanal from I, II, and III, respectively (Table 5). These homologs were therefore, respectively, two and one methylene units ($-CH_2-$) shorter than III. Sites of unsaturation in all homologous triolefins appeared to be (1,7,13). Chromatograms of ozonolysis products of III were complicated by the presence of

TABLE 4. PENTACOSATRIENE (III) IN EXTRACTS OF STABLE FLIES FROM DIFFERENT LOCATIONS AND SEASONS[a]

| Condition of flies[b] | Micrograms found per fly[c] |
|---|---|
| Females | |
| Gainesville summer | 0.0543 |
| Gainesville winter | 0.0039 |
| St. Croix March | >0.001 |
| Males | |
| Gainesville summer | 11.86 |
| Gainesville winter | 12.27 |
| St. Croix March | 14.89 |
| Spermathecae[d] | none |

[a] Determined by GC on 1.8 m × 2 mm ID glass column of 5% SE-30 on 124–140 mesh Gas Chrom Q.
[b] Sexed within 24 hr of eclosion, reared to 5 days old, ca. 1000 flies.
[c] Extracts combined as in "Total" in Table 1.
[d] Determined by capillary GC on 15 m × 0.23 mm DB-1 (trace of KI3000 observed).

TABLE 5. OZONOLYSIS FRAGMENTS OF NATURAL TRIOLEFINS DETECTED BY GC[a]

| Aldehydes observed | Triolefin material ozonized (% composition) | | |
|---|---|---|---|
| | $C_{25}$ | $C_{24}$ | $C_{23}$[b] |
| $C_7$ | 0 | | |
| $C_8$ | 0 | | |
| $C_9$ | 4.4 | | 8.3 |
| $C_{10}$ | 0 | minor | 79.2 |
| $C_{11}$ | 1.1 | major | |
| $C_{12}$ | 88.3 | | 12.5 |
| $C_{13}$ | 0.9 | | |
| $C_{14}$ | 0 | | |
| $C_{15}$ | 2.2 | | |
| $C_{16}$ | 3.1 | | |
| $C_{17}$ | 0 | | |
| $C_{18}$ | 0 | | |
| | 100 | | 100 |

[a] Analyzed on 3% SE-30 (1.8 m × 2 mm ID) glass column on 120–140 mesh Chromosorb W AW-DMCS.
[b] Analyzed on 5% Carbowax 20-M (1.8 m × 2 mm ID) s.s. column on 100–120 mesh Chromosorb W AW.

several dialdehydes. When a polar column (HI-EFF-1BP, modified DEGS) was used for CI-MS studies, the presence of large quantities of $C_6$ dialdehyde was confirmed. Dialdehydes prepared by ozonolysis of synthetic unsaturated aldehydes and alicyclic olefins were used as standards for comparison. Prominent ions seen in the methane CI-MS spectra of aldehydes were those for M + 1, M + 29, M + 41, M + 1 − 18, and M − 1 − 18, the first three ions resulting from the addition of $H^+$, $C_2H_5^+$ and $C_3H_5^+$, and the latter two ions due to loss of water. Dialdehydes showed additional prominent ions at M + 1 − 36 and M − 1 − 36 from loss of a water molecule from each end of the molecule. Distinction between mono- and dialdehydes was obtained by printing out the spectrum of each peak. Reconstructed mass chromatograms for the ions of M + 1 − 18 and M − 1 − 18 showed which compounds were monoaldehydes, as these peaks had no fragments at M + 1 − 36 or M − 1 − 36.

It appeared that III was chemically identical to the only polyene, (*Z*,*Z*)-1,7,13-pentacosatriene, described by Sonnet et al. (1977), as the major ozonolysis products, dodecanal and hexandial, were the same.

## DISCUSSION

*Quantitation of Polyolefins.* Experments in repeated extraction showed that surface washes alone removed about 70% of III from either sex. Quantities

listed in Table 1–4 were not corrected for total extraction. At emergence, quantities of III were very low and roughly the same for males and females, but thereafter production in males greatly exceeded that in females. For flies reared in complete isolation, 5-day-old females produced only 0.2% as much III as males. A "primer" effect due to the presence of males was not responsible for the production of III in females, in the absence of direct physical contact, as shown by its absence in females that were semiisolated. Male and female flies reared in complete isolation produced 29 and 8% as much III, respectively, as those sexed at 24 hr and reared with consexuals as virgins. When flies were reared together, there was physical transfer of III from males to females. Note that the highest level of III was produced by semiisolated males which could see but not contact females. Harris et al. (1976) also reported some transfer of an unidentified $C_{25}$ hydrocarbon (produced by mature males) to female flies during a single mating and more transfer of this material if females were held 24 hr with males.

Mature virgin males had similar quantities of III (12 $\mu$g, 95%) and small amounts of other polyolefins, regardless of their origin (Table 4), whereas the quantities of I and II were usually 0.15 $\mu$g (1.2%) and 0.26 $\mu$g (2.1%), respectively, and quantities of higher homologs were similar to I and II (Table 2). Virgin females had much smaller quantities of III, although those reared in summer had 12 times more III than those reared in winter in Gainesville (54 ng vs. 4 ng). We had expected to see a much larger increase in the level of polyolefins in virgin females with the onset of summer, but this was not observed. It must be noted that accidental inclusion of one male bearing 12 $\mu$g of III would raise the quantity in corresponding females by 4 ng/female and that extracts of 3000 winter-reared females had a total of 12 $\mu$g of $C_{25}$ triolefin, the same quantity as one male. The flies were therefore carefully sexed two times, once at 24 hr and once after freezing. We found (fall 1976 and spring 1976) that the *trans* olefin and polyolefin content of female extracts were very small, as opposed to the quantities reported by Muhammed et al. (1975), and that no attraction was observed to any fractions with male flies in olfactometer tests. Bioassays of polyolefins and separated components recovered from females, mixed sex polyolefins, and three synthesized polyolefns described in this report, using the equipment and procedures of Muhammed et al. (1975) for attraction of males, gave negative results; also, no evidence was seen for biological activity of these materials in females (J.W. Mackley, unpublished data).

Females described as "unmated" (Table 3) are often described as "virgin" because they are not inseminated. However, these results suggest that physical contact is occurring since male-derived III appears exclusively on abdomens of "unmated" females that were sexed at 18 hr and held for 3 days. The mated females have spread III over all body parts, with proportions similar to those found in males. It is interesting to note that most was found on legs in older flies.

This process may be general in Diptera, as male tsetse flies of several species have been shown to transfer species-specific, male-exclusive long-chain alkenes to females of the same species upon mating (D.A. Carlson, unpublished data).

We feel that the differences in quantitation of III between our present results and those reported by Muhammed (1975) are the results of physical contact between sexes before the flies were knocked down at 18 or 24 hr for sexing. Less likely factors include careless sexing, seasonal changes (Table 4), loss of the original colony of flies at the University of Florida used by Muhammed (1975) that necessitated use of the USDA colony, a different strain being reared at a different location, or dietary changes involving the removal of antibiotic dosages from blood. Other factors that could have affected the strains differently include temperature and relative humidity of adult and larval maintenance, fly density in cages, larval rearing media, and methods of handling. The sex pheromone of a grass grub beetle (Scarabaeidae) has been directly linked to symbiotic bacteria (Hoyt et al., 1971), while Brand et al. (1975) observed bacterial conversion of α-pinene to *trans*-verbenol found in frass of a bark beetle (Scolytidae) *Ips paraconfusus*. House fly (*Musca domestica* L.) feces contain the hydrocarbon attractant (Z)-9-tricosene produced by cellular processes especially in mature female flies (Dillwith et al., 1981). However, it is possible that the presence of antibiotics in the blood diet of stable flies could cause decline in a bacterium-produced attractant. Preliminary tests to determine the effect of antibiotics on polyolefin production in females were inconclusive.

If the small quantities of polyolefin in extracts of females derived solely from males, it arrived in females through body contact before sexing at 24 hr. The female material should then be identical to male material, which should preclude its being a male attractant. If the triolefin fraction from the female contains an undiscovered sex attractant, it would have to be a uniquely potent material because there is such a small titer of any triolefin in mature virgin females. In any case, the minute quantity of $C_{25}$ triolefin from females appeared to be chemically identical to that from males.

The proposition that male-produced mating deterrents against males are involved in the sexual communication schemes of several diptera is intriguing (Schlein et al., 1981). However, only one compound has been identified in Diptera that has an antiaphrodisiac function, as *cis*-vaccinyl acetate is produced by male *Drosophila melanogaster* and transferred to the female during mating (Jallon et al., 1981). We therefore investigated the components of the enlarged spermathecal duct of mature male stable flies and found small amounts of hydrocarbons in the crude extract, but no III.

Accessory-gland extracts from blood-fed male stable flies have been injected into females, and these receptivity-inhibiting substances prevented insemination at as low as 0.25 gland equivalents. The secretions were studied for their effect upon oviposition, fecundity and insemination in females. These receptiv-

ity-inhibiting substances were not found in sugar-fed males and were not identified (Morrison et al., 1982). However, we have no evidence that III is the responsible material.

## CONCLUSION

Three triolefins identified from male stable flies were (*Z*,*Z*)-1,7,13-tricosatriene (I), (*Z*,*Z*)-1,7,13-tetracosatriene (II), and (*Z*,*Z*)-1,7,13-pentacosatriene (III). Molecular weights were obtained for several homologous trienes. Synthesized compounds (I–III) appeared to be identical to natural materials.

No evidence was obtained by bioassay to suggest that polyolefins observed in females or the synthesized compounds were attractive to males (Mackley, unpublished data). The small, but real, amount of (III) in unmated females appeared to be chemically identical to that present in males in much higher quantity, and small amounts of higher trienes were observed in both sexes. The differences in data in the tables are natural (i.e., variation among different batches of flies). The effects of seasonality, strain, "priming," geographic location, and age on the appearance of III in females are not significant enough to account for the large quantities reported in virgin females by Muhammed et al. (1975). We conclude that III found in virgin females in that study was derived from males before sperm transfer. Female stable flies do produce some III and other polyolefins, although the quantities found were small fractions (1/122 to 1/3000) of that produced by males upon maturation.

Alternatively, this material may be a primer used to stimulate the female to mate, as suggested by Harris et al. (1976), or an antiaphrodisiac that inhibits further mating with once mated females. We concluded that III is not transferred to females by genital contact alone in stable flies but that it is transferred by another route and that its function is unknown. However, the mechanism of transfer of large quantities of III to females is unknown, as is its biological origin. If a sex attractant is present in stable fly females, it does not appear likely that is a material described here.

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# SOLVENT EFFECTS ON CONFORMATION OF A SEX PHEROMONE OF *Cydia* (*Laspeyresia pomonella* L.; LEPIDOPTERA: TORTRICIDAE: OLETHREUTINAE), CODLEMONE (8,10-DODECADIENOL)

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**Abstract**—The conformations of codlemone (8,10-dodecadienol) and of a model analog have been studied by CNDO calculations. Stable conformations are found for the folded forms when the polarity of the solvent is taken into account.

**Key Words**—Conformations, solvent, folded forms, codlemone, *Cydia*, *Laspeyresia pomonella* L., Lepidoptera, Tortricidae, Olethreutinae.

By studying a series of sex pheromones of various species of Lepidoptera, several groups have shown experimentally (Priesner et al., 1975; Blum et al., 1975; C. Descoins, E. Priesner, and M. Lettere, unpublished results) that certain structural features play an essential part in their biological activity. It is claimed that the distance between the terminal functionality and the site of unsaturation, as well as the nature of the carbon fragment beyond the unsaturation, are of prime importance

$$CH_3(CH_2)_n-(CH=CH)_p-(CH_2)_m-X$$

Several theories have been proposed to account for these experimental results (Kafka and Neuwirth, 1975; Kikuchi, 1975).

In the model of Kafka and Neuwirth (1975), the pheromone or its analog is assumed to interact with its receptor in a conformational state of lowest energy. The authors conclude that the binding strength between pheromone and



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receptor is well expressed by the dipole moment and the polarizability of the subsites (terminal function, unsaturation, and the alkyl group beyond the unsaturation).

Kikuchi (1975), in a statistical study of the distances between these subsites in large samples of conformers (100 for each molecule), has established a correlation between these distances and the electrophysiological activities of the molecules.

We have adopted a different approach, starting from a hypothesis which was inspired by the study of flexible alkylated chains (Breslow et al., 1978; Tchapla and Fabre, 1982): A carbon chain of sufficient length tends to fold in a polar solvent. This lowers the energy of the solvent and also that of the molecule if there are functional groups which can interact favorably. In particular, we examined the interaction between a terminal functional group (OH) and carbon–carbon double bonds located some distance away on the chain. Because of the size of the system under study, we are forced to use semiempirical methods. Ab initio calculations on such a system are virtually impossible with present day computer facilities.

We have performed CNDO calculations on different conformations of codlemone: (1) the "extended" codlemone which corresponds to the completely staggered conformation as considered by Kafka and Neuwirth (1975) (see Figure 1), and (2) several "folded" conformations (Figure 1). In the folded structures, the distances between the two "functional" groups (the H of the OH group and

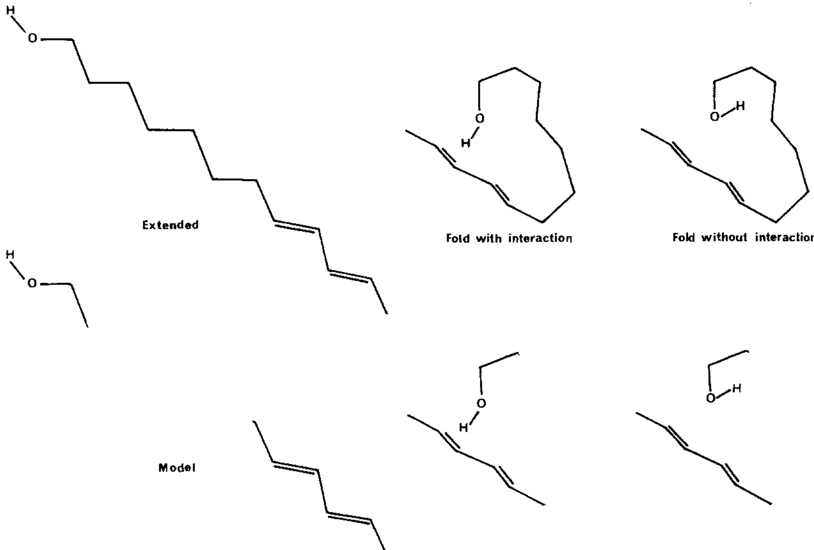


FIG. 1. Conformations of codlemone and a model.

TABLE 1. CODLEMONE ELECTRONIC ENERGY (IN eV)

| Dielectric constant of the solvent | Extended codlemone | Folded with interaction | Folded without interaction |
|---|---|---|---|
| $\epsilon = 0$ | −3283.022 | −3282.697 | −3282.839 |
| $\epsilon = 36$ | −3284.067 | −3284.574 | −3283.902 |

the middle of the $C_{10}$–$C_{11}$ double bond) have been found to be in the range of 2.4–2.6 Å. Only the results of the most stable conformation, in which H–$C_{10}$ = 2.533 Å and H–$C_{11}$ = 2.474 Å, are presented in Table 1.

This geometry can be understood by examining the frontier orbital interactions between the H orbital of the OH group and the HOMO of the butadiene system. Because the HOMO is antisymmetric, the best overlap with the symmetric H 1*s* orbital is reached when the H interacts with only one of the two double bonds. As $C_{10}$ and $C_{11}$ are in phase, the H is situated between the two carbons but most closely approaches the terminal carbon ($C_{11}$) which has the larger coefficient. The $C_8$–$C_9$ double bond is less favored because it requires a more strained ring.

In order to examine the influence of the solvent polarity, we performed a CNDO calculation, using the modified version of Constanciel and Tapia (1978), in which the dielectric constant $\epsilon$ is introduced to take into account the solvation effects: a virtual charge model accounts for the solvent polarization. The solvation energy of an ion of radius $a$ and charge $Q_0$ is given by the formula

$$-(1 - \epsilon^{-2})\, Q_0^2/2a$$

The value $\epsilon = 36$ has been chosen to represent a polar physiological medium (sensory liquid). The analogy between the sensory liquid and dimethyl formamide ($\epsilon = 36$) has already been suggested by Dr. Kaissling (C. Descoins, private communication). The same qualitative conclusions are reached if one considers larger $\epsilon$ values.

The "folded" conformation is found stable with respect to the "extended" one when a polar solvent is used ($\epsilon = 36$), whereas the "extended" conformation is favored for the apolar solvent ($\epsilon = 0$). In the folded conformation, the charge transfer which occurs between the OH group and the double bond develops larger net charges on each of them. This conformation is therefore more stabilized by the introduction of the solvent polarity.

In order to demonstrate the role of the through-space interaction between the two functional groups, we turned the OH away from the C–C double bond. This cancels the interaction without introducing any other change in the conformation of the codlemone. It corresponds in Table 1 to the column titled "folded without interaction." In a polar solvent ($\epsilon = 36$), the "folded conformation with

interaction" is found to be −0.672 eV more stable than the "folded conformation without interaction." The reverse is true for $\epsilon = 0$ (+0.142) because the OH rotation leads to an unfavorable eclipsed conformation. The "folded conformation without interaction" is less stable that the "extended" conformation for $\epsilon = 0$ as well as for $\epsilon = 36$.

That the interaction was through space and did not result from the distortion of the carbon skeleton, was also verified by studying a model analog. We calculated the intermolecular interaction between an alcohol (ethanol) and a diene (1,4-dimethyl-1,3-butadiene), the "functional" atoms being positioned in the same manner as in the folded codlemone. The expressions "extended" and "folded with (or without) interaction" refer here to the geometrical analogue of the codlemone (Figure 1). The results of these calculations appear in Table 2.

For the folded interacting model (ethanol–1,4-dimethyl-1,3-butadiene), a significant stabilization is only observed for a polar solvent ($\epsilon = 36$) and for the geometry where the H orientation allows the interaction (see Table 2). The stabilization relative to the "extended" conformation is $\Delta E = -0.737$ eV. It is comparable to that observed for codlemone. As was also the case for codlemone, this stabilization is too small, for an apolar solvent ($\epsilon = 0$) to overcome the destabilization due to the OH rotation. A significant proportion of the stabilization of codlemone can therefore be attributed to an interaction through space between the two functional groups.

The same calculation method was used to study another analog of codlemone, chosen among a series of molecules synthesized in the laboratory of C. Descoins. Its physiological activity has been determined by electroantennogra-

TABLE 2. ELECTRONIC ENERGY (eV) OF ETHANOL–1,4-DIMETHYL-1,3-BUTADIENE SYSTEM[a]

| | Extended | Folded with interaction | Folded without interaction |
|---|---|---|---|
| Isolated ethanol | | | |
| $\epsilon = 0$ | −1013.640 | −1013.480 | −1013.484 |
| $\epsilon = 36$ | −1014.631 | −1014.501 | −1014.505 |
| 1,4-Dimethyl-1,3-butadiene | | | |
| $\epsilon = 0$ | −1362.518 | −1362.518 | −1362.518 |
| $\epsilon = 36$ | −1362.535 | −1362.535 | −1362.535 |
| Ethanol–1,4-dimethyl-1,3-butadiene system | | | |
| $\epsilon = 0$ | −2376.144 | −2376.050 | −2376.006 |
| $\epsilon = 36$ | −2377.146 | −2377.883 | −2377.211 |

[a]The different energy values of the isolated ethanol correspond to a conformation effect.

phy (results of Dr. E. Priesner, whom we thank): The *E*6,*E*8-decadienol is 1000 times less active than the natural pheromone. (This activity is expressed in equipotent doses of stimulus on a semilogarithmic scale). A stable folded conformation was not found for this analog. Because of the strain it would introduce, the folding of the carbon chain does not allow the OH and the diene to come near enough for a favorable interaction to take place.

The results of this study point out the importance of the effect of the solvent on the conformation of this type of pheromone and of flexible analogs. Based on our calculations, we conclude that the solvent effect imposes a conformational change from an "extended" structure to a "folded" one, so that the functional groups can interact. We think it necessary to take into account such folded conformations in any attempt to explain the interactions between pheromones and their receptors. Only a knowledge of the receptor structure would clarify the necessity of the pheromones to be folded.

*Acknowledgments*—We thank Professor C. Descoins (INRA) for introducing us to the field of pheromones and for helpful discussions.

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# BEHAVIORAL BIOASSAYS OF TERMITE TRAIL PHEROMONES

## Recruitment and Orientation Effects of Cembrene-A in *Nasutitermes costalis* (Isoptera: Termitidae) and Discussion of Factors Affecting Termite Response in Experimental Contexts

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**Abstract**—The monocyclic 14-membered ring diterpene, cembrene-A, previously identified as a nasutitermitine trail pheromone, was tested for its effectiveness as a trail pheromone in *Nasutitermes costalis*. Artificial trails prepared from serial dilutions of racemic cembrene-A over a concentration range of $10^{-1}$-$10^{-6}$ mg/ml were ineffective in recruiting termites. Serial dilutions of racemic cembrene-A ranging in concentration from $10^{-1}$ to $10^{-5}$ mg/ml produced an orientation effect. Chiral cembrene-A produced recruitment in soldiers at $10^{-1}$ and $10^{-3}$ mg/ml and was less ineffective in recruiting workers. Soldiers always showed a lower and more variable recruitment response to chiral cembrene-A than to sternal gland extracts. The behavioral response to both chiral and racemic cembrene-A was different in quantity and quality from that observed for sternal gland extract. Based on the results of these behavioral tests, cembrene-A appears to be a generalized nasute orientation pheromone which may show recruitment properties at unnaturally high concentrations.

**Key Words**—*Nasutitermes costalis*, Isoptera, Termitidae, termite, trail pheromone, orientation, recruitment, cembrene-A, chemical communication.

## INTRODUCTION

The extreme reliance on chemical signals in the coordination of social activities such as defense, foraging, and caste regulation makes termites interesting sub-



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jects for the analysis of the chemistry of communication. Several research groups have attempted to isolate and chemically characterize termite trail pheromones (Moore, 1966; Hummel and Karlson, 1968; Matsumura et al., 1968; Birch et al. 1972; reviewed in Prestwich, 1983). Our recent studies have shown that the trail pheromone of *Nasutitermes costalis* is characterized by an ephemeral component that regulates recruitment and a persistent substance that serves as a long-lasting orientation cue (Traniello, 1982a; Traniello and Busher, 1985). Birch et al. (1972) identified the trail substance of *N. exitiosus* as a single compound, cembrene-A, and other studies (Kaib et al. 1982; Oloo and McDowell, 1982; McDowell and Oloo, 1984) suggest that this compound may serve as the trail pheromone or as a component of the trail pheromones of other termitid species. However, we wondered if a single compound was sufficient to produce both the ephemeral recruitment and persistent orientation effects that appear to characterize nasute termite responses to their trail pheromones and if Old and New World nasutitermitines use similarly structured trail substances. We tested the effectiveness of racemic and chiral forms of cembrene-A as recruitment and orientation pheromones in *N. costalis* using a behavioral bioassay that distinguishes between orientation and recruitment effects.

## METHODS AND MATERIALS

*N. costalis* workers and soldiers were obtained from a vigorous colony collected in the Lesser Antilles in 1973 and maintained in the insectary at Boston University as described in Traniello and Busher (1985). Synthetic racemic cembrene-A dissolved in hexane at 1 mg/ml and chiral cembrene-A [*r* (−)-enantiomer] purified from extracts of cephalic glands of *Cubitermes glebae* (Prestwich, 1984) were supplied by Prof. Glenn D. Prestwich and Mr. Ambarish K. Singh of the State University of New York at Stony Brook. We tested these particular forms because of their availability; the optical rotation of cembrene-A identified as the trail pheromone of *N. existiosus* (Birch et al., 1972) has not been determined, nor has it been determined for any other nasute species trail pheromones. Both compounds were freshly serially diluted 10:1 in hexane and kept in glass on ice throughout the tests. Fresh lots of cembrene-A were used for most experiments. A stock solution of 1 mg/ml of racemic cembrene-A, stored at 4°C for over six months, retained a purity of 50%. Degradation products comprised the remaining 50%.

Extracts of large third-instar worker (LW3) sternal glands were prepared in hexane following Traniello and Busher (1985). Several glands were pooled to decrease individual variation. All sternal gland extracts tested were standardized at a concentration of 1 gland per 10 $\mu$l of hexane.

*Distinguishing between Behavioral Responses of Recruitment and Orientation.* We strongly believe that termites, as well as other social insect species

such as ants, show different biologically meaningful and experimentally discernible responses involved in trail communication. Without any teleological implication, we term these responses recruitment and orientation, consistent with the terminology currently used to describe analogous behaviors in ants (e.g., Hölldobler and Wilson, 1970). We feel that these distinctions are critical to the development of bioassays required for trail pheromone analysis.

Recruitment is a process that mediates the communication of information about the location of a food source, new nest site, or a source of disturbance. During this process, nestmates are mobilized from the nest to the target area. A chemical signal, in the absence of any other stimuli, is often sufficient to induce this mobilization. Recruitment pheromones with mass communication properties regulate excitation and attraction in addition to orientation. One compound may have both functions, but the literature suggests that the secretions of more than one exocrine gland may be responsible for producing trail pheromones that code for either excitation or orientation. The excitatory chemical is often ephemeral, serving as a regulator of recruitment, while the orientation chemical is more durable. The latter pheromone serves only as a guide and is unable to stimulate individuals to leave the nest. Traniello (1982a) has described the role of these two processes in trail communication in *Nasutitermes costalis* and has discussed their significance.

*Bioassay for Recruitment and Orientation.* Bioassays for recruitment and orientation were performed on groups of termites of fixed and equal numbers of workers and soldiers placed on filter paper under Petri dish covers (100 mm diameter). We recognize that the caste ratio and nest arrangements are artificial in these test groups, but are necessary to bioassay the properties of different compounds. A minimum of 100 individuals of each caste was put into each test group. In order to maintain a constant soldier–worker ratio, each group was used for only two or three trials. Artificial trails were offered on filter paper at a point along the circumference of the Petri dish where termites were clearly active. Recruitment and orientation bioassays were performed as described in Traniello and Busher (1985). A racemic cembrene-A trail (10$\mu$l per 10 cm) was tested for its effectiveness as a recruitment pheromone during a 3-minute period. We recorded a positive recruitment response for individual termites if the artificial trail tested had the ability to draw termites out from a test group and cause them to follow for at least 5 cm. Immediately following this test, a sternal gland extract trail (at a concentration of one LW3 sternal gland per 10 $\mu$l of hexane per 10 cm) was drawn, and the same test group was tested as a control to assure that the termites were capable of a recruitment response (Figure 1a). Comparison of recruitment response to racemic cembrene-A and hexane (solvent) was performed in a similar manner. Recruitment to chiral cembrene-A was tested by drawing sternal gland and cembrene-A trails simultaneously but separated by approximately 10 cm (1/3 the circumference of the Petri dish).

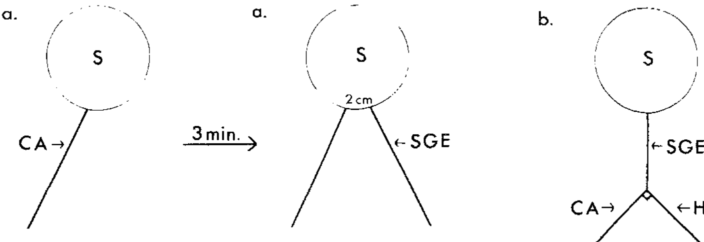


FIG. 1. Schematic representation of bioassay conditions for recruitment and orientation tests. The recruitment test (a) consisted of offering a cembrene-A trail, recording response for 3 min, then immediately offering a sternal gland extract trail and recording response for an additional 3 min (a′). The orientation test (b) consisted of offering a single sternal gland extract trail. At its terminus, cembrene-A and solvent (hexane) trails were drawn. Response was recorded for 3 min. All trails were 10 cm in length but are not drawn to scale. S = test group, SGE = sternal gland extract trail, CA = cembrene-A trail, H = hexane, solvent trail.

Orientation tests were conducted in two ways using a Y-shaped configuration (Traniello and Busher, 1985) (Figure 1b). First, termite test groups were placed at the beginning of the sternal gland extract trail. The lid was carefully raised, and the termites were allowed to leave the nest and recruit to the sternal gland extract trail. This we refer to as a test conducted under "undisturbed" conditions. In the second test, the same Y-choice arrangement was employed, but termites were given access to the sternal gland extract stem of the Y by shaking them from test groups onto the beginning of the trail. This we term a "disturbed" condition test. Although at first highly agitated, they quickly followed the sternal gland extract trail to its terminus where they could choose to follow a cembrene-A or solvent trail. We acknowledge the unnatural quality of this test and believe that creating this temporary disturbance situation decreases the threshold level that must be exceeded for soldiers and workers to search for and follow chemical trails. We therefore used the test to examine the effect of disturbance on trail-following behavior. In all orientation trials, a minimum of 100 individuals of each caste reaching the junction were tested for every concentration of cembrene-A.

*Statistical Analysis.* Means and standard deviations are reported in the text and in the tables. Although we considered an individual to show a positive response to a trail when it followed for more than 5 cm, the response to a trail of a given composition is considered positive when a series of replicates resulted in a level of response in which the coefficient of variation was less than or equal to 150%. This allows us to discriminate between a low mean response (with high variance) and a high mean response (with high variance). A low mean is considered a response of less than two individuals in a series of replicates. The Mann-Whitney U test was used to compare responses of both castes to each

compound and to different experimental conditions. Kruskal-Wallis tests were performed to distinguish differences between the responses at varying concentrations. Responses are reported as percentages of termites tested (those reaching the junction and perceiving the cembrene-A) in the orientation tests. Tests for the equality of two percentages were calculated to distinguish differences between caste response and treatments (Sokal and Rohlf, 1969).

## RESULTS

*Recruitment Response to Racemic Cembrane-A.* The results of the sequential tests of six concentrations of cembrene-A are given in Table 1. Although mild recruitment of soldiers by the racemic cembrene-A trail was observed, if indeed the responses recorded represent recruitment and not simply a following response of highly motivated individuals, the average response was less than two individuals for each concentration tested. Recruitment of soldiers to racemic cembrene-A was significantly greater in trials of cembrene versus hexane (mean = 0.5 ± 0.9, $N$ = 60) than in trials of cembrene versus sternal gland

TABLE 1. RECRUITMENT TO STERNAL GLAND EXTRACT AND RACEMIC CEMBRENE-A TRAILS USING TEST GROUPS OF 100-INDIVIDUALS OF EACH CASTE[a]

| Racemic cembrene-A | | | | |
|---|---|---|---|---|
| | Response | | Sternal gland control response | |
| Concentration (mg/ml) | Soldiers | Workers | Soldiers | Workers |
| $10^{-1}$ | 0.6 ± 1.1 | 0.0 | 8.0 ± 2.4 | 2.0 ± 1.5 |
| $10^{-2}$ | 0.5 ± 1.1 | 0.0 | 8.4 ± 2.9 | 2.1 ± 1.4 |
| $10^{-3}$ | 0.4 ± 0.7 | 0.0 | 7.9 ± 3.5 | 2.6 ± 1.7 |
| $10^{-4}$ | 0.3 ± 0.7 | 0.0 | 7.6 ± 1.8 | 1.8 ± 1.3 |
| $10^{-5}$ | 0.5 ± 1.1 | 0.0 | 6.2 ± 4.9 | 1.2 ± 2.1 |
| $10^{-6}$ | 0.5 ± 0.7 | 0.0 | 6.8 ± 3.7 | 1.9 ± 1.4 |
| | | | Hexane (solvent) control response | |
| | | | Soldiers | Workers |
| $10^{-1}$ | 0.9 ± 1.0 | 0.0 | 0.0 | 0.0 |
| $10^{-2}$ | 0.8 ± 0.9 | 0.0 | 0.0 | 0.0 |
| $10^{-3}$ | 1.5 ± 1.3 | 0.1 ± 0.3 | 0.0 | 0.0 |
| $10^{-4}$ | 1.5 ± 1.3 | 0.4 ± 0.5 | 0.0 | 0.0 |
| $10^{-5}$ | 0.6 ± 0.7 | 0.0 | 0.0 | 0.0 |
| $10^{-6}$ | 0.3 ± 0.5 | 0.0 | 0.0 | 0.0 |

[a] The means and standard deviations represent 10 trials at each concentration of cembrane-A.

extract (mean = 0.7 ± 1.0, $N$ = 60; Mann-Whitney U, $P \leq 0.01$). However, recruitment to racemic cembrene-A was always significantly less than recruitment to sternal gland extract (Mann-Whitney U for each concentration, $P \leq 0.01$). Although statistical tests yield significant differences between various treatments, these results can be explained by the consistently zero response in one treatment being compared to a low but nonzero response of another. The difference between zero and one may be statistically significant but probably has no biological importance. Workers were very seldom recruited by a racemic cembrene-A trail, and a total of only five individuals in 120 trials were recorded. Trails prepared from sternal gland extracts were consistently effective in recruiting both soldiers and workers in the pattern previously described by Traniello (1981, 1982a,b) and Traniello and Busher (1985).

*Recruitment Response to Chiral Cembrene-A.* The results of recruitment bioassays to chiral cembrene-A are given in Table 2. Due to a lack of material, only three concentrations of chiral cembrene-A ($10^{-1}$, $10^{-3}$, and $10^{-5}$ mg/ml) were tested. Recruitment was observed in soldiers at $10^{-1}$ and $10^{-3}$ mg/ml. However, responses were lower on average at $10^{-1}$ mg/ml than those observed to sternal gland extracts and were significantly lower at $10^{-3}$ mg/ml ($P \leq 0.05$). Recruitment to chiral cembrene-A was significantly higher than response to racemic cembrene-A ($P \leq 0.001$), but at $10^{-5}$ mg/ml recruitment ceased. All responses were highly variable with coefficients of variation ranging from 65% to 100%. This is a much greater variation than seen in recruitment to sternal gland extract in which the overall coefficient of variation was 46%.

Worker responses to chiral cembrene-A were significantly lower than to sternal gland extract at all concentrations ($P \leq 0.01$). There is no significant difference between worker response to chiral cembrene-A and racemic cembrene-A. There were no significantly different responses for workers among the concentrations of chiral cembrene-A (Kruskal-Wallis test, = 0.191).

In summary, recruitment to chiral cembrene-A in soldiers occurs at very high concentrations ($10^{-1}$ and $10^{-3}$ mg/ml), is highly variable, and ceased at

TABLE 2. RECRUITMENT RESPONSES TO CHIRAL CEMBRANE-A TRAILS IN TEST GROUPS OF 100 INDIVIDUALS OF EACH CASTE[a]

| Chiral cembrene-A | | | |
|---|---|---|---|
| Concentration (mg/ml) | Soldiers | Workers | $N$ |
| $10^{-1}$ | 8.2 ± 8.2 | 1.3 ± 2.8 | 12 |
| $10^{-3}$ | 5.2 ± 3.4 | 0.8 ± 1.6 | 16 |
| $10^{-5}$ | 0.0 | 0.0 | 7 |

[a] Means, standard deviations, and the number of trials ($N$) are given for each concentration tested.

$10^{-5}$ mg/ml. For a series of replicates, workers did not exhibit a positive recruitment response (coefficient of variation > 150%), although individuals occasionally did so.

*Control Bioassays of Sternal Gland Extract Preparations.* In assays of sternal gland extract preparations made prior to any comparison with cembrene-A or chiral cembrene, the average recruitment response was 9.4 ± 6.1 soldiers and 2.7 ± 4.0 workers ($N = 11$). There is no significant difference between sternal gland extract recruitment of soldiers or workers tested prior to or during tests of both cembrene compounds (Mann-Whitney U test, $P = 0.45$). Recruitment and orientation of soldiers or workers to hexane (solvent) was never observed.

*Orientation Effects of Cembrene-A.* Artificial trails of racemic cembrene-A ranging in concentration from $10^{-1}$ to $10^{-5}$ mg/ml provided orientation cues for termites (Tables 3 and 4). At concentrations of $10^{-5}$ and $10^{-6}$ mg/ml significantly more soldiers ($ts = 2.28$, $P = 0.023$; $ts = 13.47$, $P < 0.0001$, test for equality of two percentages) and workers ($ts = 6.27$, $P < 0.0001$; $ts = 11.50$, $P < 0.0001$) followed the racemic cembrene-A trail in the disturbed situation than under undisturbed conditions. The increase in trail-following responses may be due to lower following thresholds of termites shaken from test groups. At concentrations of $10^{-5}$ mg/ml in undisturbed conditions and $10^{-3}$ mg/ml in disturbed conditions, significantly more soldiers than workers used racemic cembrene-A trails as an orientation cue ($ts = 3.38$, $P = 0.001$; $ts = 3.68$, $P < 0.001$, respectively).

## DISCUSSION

Cembrene-A has been identified as the trail pheromone of *Nasutitermes exitiosus* (Moore, 1966; Birch et al., 1972), and there is evidence suggesting that it is present in other species. Oloo and McDowell (1982) have reported that

TABLE 3. ORIENTATION TO RACEMIC CEMBRENE-A TRAILS IN DISTURBED CONDITION[a]

| Cembrene-A concentration (mg/ml) | Soldiers | | Workers | |
|---|---|---|---|---|
| | No. tested | % orienting | No. tested | % orienting |
| $10^{-1}$ | 103 | 63.1 | 101 | 71.3 |
| $10^{-2}$ | 101 | 51.5 | 133 | 48.1 |
| $10^{-3}$ | 113 | 79.6 | 131 | 58.0 |
| $10^{-4}$ | 114 | 63.2 | 139 | 59.7 |
| $10^{-5}$ | 115 | 44.3 | 146 | 45.2 |
| $10^{-6}$ | 128 | 59.4 | 109 | 50.5 |

[a] Results are presented as percentages of termites tested.

TABLE 4. ORIENTATION TO RACEMIC CEMBRENE-A TRAILS IN UNDISTURBED CONDITION.[a]

| Cembrene-A concentration (mg/ml) | Soldiers | | | Workers | | |
|---|---|---|---|---|---|---|
| | | % orienting | | | % orienting | |
| | No. tested | Cembrene | Hexane | No. tested | Cembrene | Hexane |
| $10^{-1}$ | 110 | 72.0 | 0.0 | 101 | 69.1 | 0.0 |
| $10^{-2}$ | 103 | 78.4 | 0.0 | 108 | 71.2 | 0.0 |
| $10^{-3}$ | 113 | 64.6 | 0.0 | 105 | 64.8 | 0.0 |
| $10^{-4}$ | 104 | 68.3 | 0.0 | 110 | 55.5 | 0.0 |
| $10^{-5}$ | 102 | 29.4 | 0.0 | 108 | 11.1 | 0.0 |
| $10^{-6}$ | 108 | 0.0 | 0.0 | 103 | 0.0 | 0.0 |

[a] Results are presented as percentages of termites tested.

*Trinervitermes bettonianus* follow extracts prepared from *T. gratiosus* trails as well as their own trails (and vice versa), and their chromatographic analyses showed that the major constituent of the trail pheromone is the same for both species (Oloo and McDowell, 1982). Recently, this constituent has been identified as cembrene-A (McDowell and Oloo, 1984). Based on the results of interspecific trail-following tests, this same component may also produce trail following in *Amitermes* and weak responses in *Macrotermes* (Kaib et al., 1982). Interpreting their results, Kaib et al. state that the "high degree of interspecific response suggests the existence of a common component or a pool of structurally very closely related components being present in the trail-active extracts tested (including the Nasutitermitinae)." cembrene-A, therefore, may be this component.

Our results demonstrate that, in the New World nasutitermitine *Nasutitermes costalis*, racemic cembrene-A is unable to produce the recruitment effects characteristic of natural trails or sternal gland extracts of this species (Traniello, 1982a, b; Traniello and Busher, 1985). Although racemic cembrene-A was not able to induce recruitment, it was used as an orientation guide and its effects were concentration related in undisturbed tests conditions. It appears that nasute termites and perhaps other termitids utilize racemic cembrene-A as the orientation component of their trail pheromones. Chiral cembrene elicited recruitment at concentrations that are probably five orders of magnitude greater than concentrations of trail pheromones that naturally occurs in termites (Birch et al., 1972). No recruitment response was recorded at $10^{-5}$ mg/ml of chiral cembrene-A. Recruitment to chiral cembrene was also more variable than recruitment to sternal gland extract. Perhaps high concentrations of an orientation compound may induce "recruitment-like" effects.

Leuthold and Luscher (1974), Tschinkel and Close (1973), Traniello (1982a,b), and Traniello and Busher (1985) have demonstrated that termite response to trails prepared from sternal gland extracts is concentration dependent: increasing pheromone concentration increases the number of termites following the trail. In our work there is no difference in recruitment to racemic cembrene-A over a concentration range of six orders of magnitude. A concentration-dependent response to chiral cembrene-A occurs only at abnormally high concentrations. This suggests that cembrene-A is not serving as a regulator of recruitment nor does it play a role in foraging organization.

Interpreting the results of studies on termite trail pheromones is sometimes difficult due to the lack of information presented on bioassays or the use of an unnatural test. Stuart (1969) and Howard et at. (1976) cautioned that the development of an unambiguous trail bioassay is crucial to the accurate behavioral or chemical identification of trail pheromones. Such a bioassay must be conducted under at least seminatural conditions which minimize disturbance and must distinguish between recruitment and orientation. "Trail following" is not a useful distinction unless the test circumstances are precisely described. Termites, when agitated and/or given limited choices, will follow trails composed of compounds known not to originate in the sternal gland. For example, we have demonstrated that extracts of the frontal gland of *N. costalis* have orientation properties, although such material is obviously not deposited as a trail pheromone (Traniello, 1981). Similarly, it is difficult to interpret results of studies of the effects of pheromone stereoisomerism (Kato et al., 1980) in recruitment and orientation of termites if behavorial bioassays are ambiguous.

Over biologically realistic concentration ranges, the principal response of *N. costalis* soldiers and workers to cembrenoid compounds is orientation. When in a disturbed condition, individuals of both castes orient to racemic cembrene-A at concentrations that range over six orders of magnitude. In undisturbed conditions, soldiers and workers show a concentration-dependent orientation response which ceases at a concentration of $10^{-6}$ mg/ml. At unnaturally high concentrations of chiral cembrene-A, a recruitment-like response is seen. However, it disappears as the concentration of the compound approaches natural levels. Bioassays based only on orientation tests or a limited range of concentrations would lead to the erroneous conclusion that cembrene-A is the trail pheromone of *N. costalis*. This further illustrates the difficulties in chemically and behaviorally identifying termite trail pheromones.

Given the discrete differences in the roles of soldier and worker castes, it is reasonable to assume that their differential response to cembrene-A may reflect these roles. The successful repulsion of an attack requires the temporary gathering of sufficient numbers of soldiers (and workers in some instances) to a specific site. Frontal gland secretions are known to mediate such defensive responses in *N. corniger* and *N. costalis* (Stuart, 1981; Traniello, 1981). It appears

that in *N. costalis* cembrene-A acts as a short-term alarm substance capable of orienting soldiers to an area of disturbance. In addition, cembrene-A may represent a common constituent of trail pheromones of nasute species, but in *N. costalis* it is not the active recruitment component.

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# ALLELOPATHIC EFFECTS OF *Citrus aurantium* L. I. Vegetational Patterning

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**Abstract**—Field observations on undisturbed stands of sour orange revealed that *Cynodon dactylon*, *Chenopodium album*, *Avena sativa*, and *Amaranthus retroflexus* were not able to grow normally and complete their life cycles under its canopies, although the same species grow well under adjacent trees of date palm. Investigations revealed that the failure of the test species to grow normally under sour orange was not due to competition for light, moisture and minerals or to differences in soil texture or pH. Soil under sour orange trees drastically reduced seed germination and/or seedling growth of test species. Aqueous extracts, decaying materials, and volatile compounds of senescent and nonsenescent sour orange leaves were found to inhibit seed germination and/or seedling growth of test species. Therefore, allelopathy appeared to be the basic factor responsible for the reduction in plant growth with competition propably accentuating its effects.

**Key Words**—Allelopathy, patterning, sour orange, *Citrus aurantium*, date palm, *Phoenix dactylifera*, *Cynodon*, *Chenopodium*, *Amaranthus*, *Avena*.

## INTRODUCTION

Several investigators have documented that allelopathy is the major cause of vegetational patterning in some forest communities (Al-Naib and Rice, 1971; Lodhi, 1975; Del-Moral and Muller, 1970). Most of such research, however, was carried out on natural forest communities and only a few investigations were made on artificial forest communities.

Sour orange, *Citrus aurantium*, grows in parks, gardens, and commercial orchards in central Iraq. Preliminary observations revealed that several herbaceous plants were not able to grow under sour orange even with repeated attempts and with adequate irrigation and fertilization. The same observations



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were also made in a *Citrus* orchard left for more than three years without any agricultural operations. Therefore this study was carried out to determine whether the failure of herbaceous plants to grow under sour orange trees was due to competition for basic growth factors (water, light, and minerals) or to allelopathy.

## METHODS AND MATERIALS

*Species Selection.* Field observations revealed that the understory vegetation in the study area is mainly dominated by *Avena sativa*, *Chenopodium album*, *Amaranthus retroflexus*, *Cynodon dactylon*, and several other minor species. The growth of the herbaceous species was observed to be considerably better under date palm, *Phoenix dactylifera*, than under sour orange.

*Physical and Chemical Analysis of Soil.* Soil moisture, reaction, texture, and selected minerals were determined to see if sour orange causes changes in those soil factors which could account for the change in vegetational patterning.

Soil moisture was determined from March to August 1983. Eight soil samples were taken randomly at the 0- to 20-cm level, weighed, oven dried for 24 hr at 105°C, and reweighed to measure the water percentage.

For physical and chemical analysis, eight soil samples minus litter were collected randomly at the 0- to 20-cm level under sour orange trees and eight under date palm trees. Each soil sample was mixed thoroughly, air dried, passed through 2-mm seives, and analyzed for pH by the glass electrode method (Piper, 1942). Soil texture was determined by mechanical analysis using a modified Bouyoucos hydrometer method (Bouyoucos, 1936; Piper, 1942). The remaining soil samples were used to determine the amount of ammonium nitrogen and nitrate nitrogen by the method of Bremner (1965), total carbon by the procedure of Anderson and Harris (1967), and available phosphorus by the method of Olsen et al. (1954). Easily extractable K, Mg, Fe, and Zn were determined by use of a Perkin Elmer atomic absorption spectrometer, model 503, according to the extraction procedure in the analytical manual supplied with the instrument.

*Shading Effect.* Light intensities were measured 50 times in different regions under sour orange and date palm trees and in full-sunlight positions. Readings were taken in July using a light meter (Photoelement S 60), and results were expressed as a percent of full-sunlight intensity.

*Effect of Field Soils on Germination and Growth of Test Species.* To determine if phytotoxins are released by sour orange trees and remain stable under field conditions, 10 soil samples were taken under sour orange trees and placed in plastic pots of 13 cm diameter. Ten other soil samples were taken under date palm trees and used as a control. Fifty seeds of each test species were planted in each pot. This experiment and all subsequent ones described below were conducted in a growth room with a 14-hr photoperiod at 1900 ft-c and 26 ±

2°C/24 ± 2°C light–dark conditions. After two weeks, seed germination was recorded and the plants were thinned to the three seedlings per pot. Oven dry weights of seedlings were taken four weeks after seeding.

*Effect of Aqueous Extracts on Test Species.* Aqueous extracts of sour orange senescent and nonsenescent leaves were prepared by boiling 10 g of freshly fallen yellow leaves or green leaves in 100 ml distilled water and then grinding in a homogenizer for 10 min. The extracts were separately suction-filtered through filter paper (15–40 μm pore size) and centrifuged at 15,000 rpm for 10 min. The solutions were made up to 100 ml with distilled water and adjusted to pH 6 (Rice, 1972). Solutions for biological activity tests were made by diluting the purified extracts to 1 : 1 [extract: nutrient Hoagland's solution (Hoagland and Arnon, 1950)]. The control solution was made in the same way except distilled water was substituted for extracts.

Twenty-five seeds of each test species were planted in separate pots, each containing 330 g of washed quartz sand. Immediately after planting, 70 ml of senescent or nonsenescent leaf test solution were added to each of five test pots. An equal amount of control solution was added to each of five control pots. All pots were watered with equal amounts of 0.5-strength Hoagland's solution alternated with distilled water. The amounts of Hoagland's solution or distilled water were adjusted to prevent any leaching. The percentage of germination was determined two weeks after planting, at which time the seedlings were thinned to the three largest seedlings per pot, allowed to grow for another two weeks, and then harvested. The biomass of roots and tops was determined on the basis of oven-dry weight.

*Effect of Decaying Sour Orange Leaves on Test Species.* Field study indicated that sour orange adds to the soil (within the top 17 cm) approximately 6 g of air-dried fallen leaves per kilogram of soil in the study area. To test the phytotoxicity of sour orange senescent and nonsenescent leaves on the test species, 50 seeds of each test species were planted separately in plastic pots containing 6 g of air-dried powder of senescent or nonsenescent leaves per kilogram of loamy soil. Equal amounts of peat moss were added to the control pots to keep the organic matter the same. All pots were watered when necessary with tap water. Seed germination was recorded two weeks after planting, at which time the seedlings were thinned to the three largest per pot, allowed to grow for another two weeks, and then harvested. Oven-dry weights of roots and tops were determined.

*Effect of Volatile Compounds from Sour Orange Leaves on* Amaranthus retroflexus. The idea used for determination of biological activities was basically that of Grove and Anderson (1981). Senescent or nonsenescent leaves (0.5, 1, and 3 g) were chopped into pieces and placed separately in the desiccator (2500 $cm^3$ size). Groups of 50 seeds of *A. retroflexus* were placed in uncovered Petri dishes (9 cm diameter) each containing 40 ml of sterilized sand and 13 ml of distilled water. Each Petri dish was placed on the perforated porcelain plate of

the desiccator, and the desiccators were tightly sealed. Accordingly only atmospheric contact existed between the chopped leaves and the test seeds. The control was subjected to the same procedure, except no chopped leaves were placed in the desiccators. Seed germination and radicle and hypocotyl lengths of all germinated seeds were measured eight days after seeding.

## RESULTS

*Physical and Chemical Properties of Soil.* The percentage of soil moisture under the sour orange trees was not significantly different from that under date palm trees (Figure 1). No significant differences were found in the pH, soil texture, or amount of any mineral elements studied under sour orange as compared with the control soil (Table 1).

*Shading Effect.* Light intensity under sour orange canopies was not significantly different from that of date palm canopies (Figure 2).

*Effect of Field Soils on Germination and Test Species.* Seedling growth of all test species except *Avena sativa* was significantly inhibited by soil collected under sour orange trees (Table 2). Soil collected under sour orange trees drastically reduced seed germination of all test species.

*Biological Activities of Aqueous Extracts.* The oven-dry weights of roots, tops, and whole plants of all test species were greatly reduced by aqueous extracts of senescent and nonsenescent sour orange leaves (Table 3). The inhibition

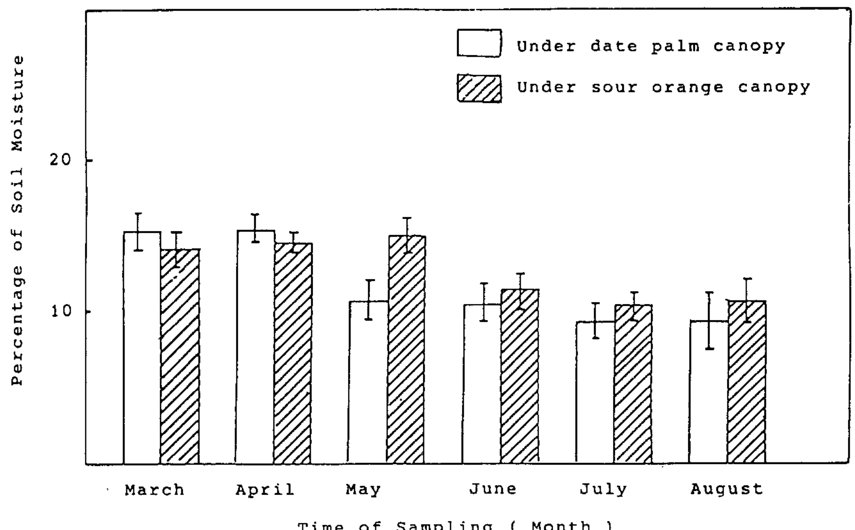


FIG. 1. Percentage of soil moisture under date palm and sour orange canopies.

TABLE. 1. COMPARISON OF SOME SOIL FACTORS UNDER SOUR ORANGE AND DATE PALM TREES

| | Stand | |
|---|---|---|
| Test[a] | Sour orange[b] | Date palm |
| pH | 7.93 | 8.08 |
| Sand (%) | 34.29 | 37.71 |
| Silt (%) | 48.31 | 50.15 |
| Clay (%) | 17.30 | 12.14 |
| C (%) | 2.73 | 2.20 |
| $NH_4$ N (ppm) | 55.30 | 46.90 |
| $NO_3$ N (ppm) | 18.90 | 13.20 |
| P (ppm) | 763.83 | 607.93 |
| K (ppm) | 62.30 | 78.75 |
| Mg (ppm) | 336.80 | 379.50 |
| Ca (ppm) | 494.00 | 521.80 |
| Fe (ppm) | 48.87 | 35.68 |
| Zn (ppm) | 1.72 | 1.74 |
| Mn (ppm) | 35.30 | 34.40 |

[a] Each value is average of eight replicates.
[b] Differences are not significant at 0.05 level.

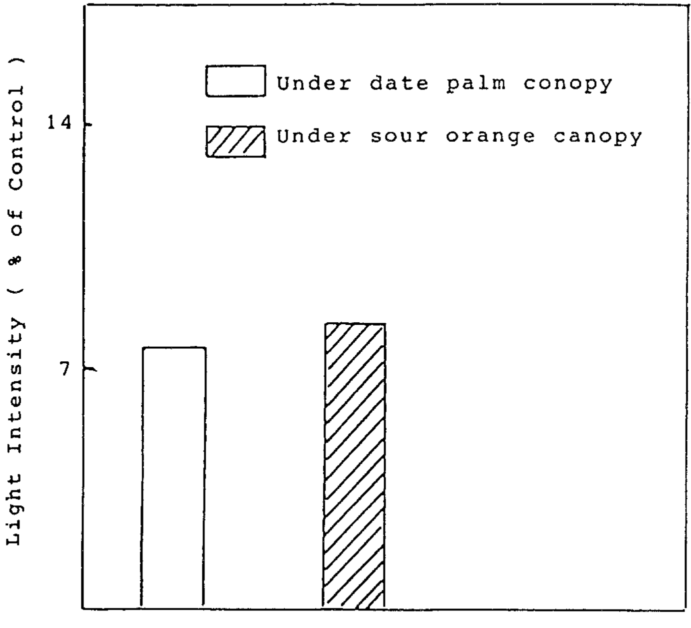


FIG. 2. Light intensity under date palm and sour orange canopies.

TABLE 2. EFFECT OF FIELD SOIL FROM UNDER SOUR ORANGE TREES ON GERMINATION AND GROWTH OF TEST SPECIES

| Species | Oven-dry weight of seedlings (mg)[a] | | Germination (% of control) |
|---|---|---|---|
| | Control | Test | |
| *Avena sativa* | 55.7 | 59.6 | 15.1 |
| *Amaranthus retroflexus* | 29.7 | 6.8[b] | 11.6 |
| *Cynodon dactylon* | 37.0 | 5.4[b] | 38.5 |
| *Chenopodium album* | 33.6 | 11.0[b] | 9.1 |

[a] Average of at least 20 seedlings.
[b] Dry weight significantly different from control at 0.05 level.

of growth by senescent leaves was more pronounced than by nonsenescent leaves for *Avena sativa* and *Cynodon dactylon* only.

Acqueous extracts of senescent and nonsenescent leaves drastically reduced seed germination of all test species except *Amaranthus retroflexus*, in which the germination was slightly inhibited by extracts of senescent leaves only.

*Biological Activities of Decaying Residues.* Decaying senescent leaves significantly reduced the growth of all test species except root growth of *Avena sativa*, top growth of *Cynodon dactylon*, and root growth of *Chenopodium album* (Table 4). The decaying nonsenescent leaves significantly inhibited the growth of all test species except top and seedling growth of *A. retroflexus*. Decaying nonsenescent leaves drastically inhibited the germination of all test species, whereas decaying senescent leaves significantly reduced germination of only three test species. Decaying nonsenescent leaves were more inhibitory to germination of *Cynodon dactylon* than senescent leaves.

*Biological Activities of Volatile Compounds.* Volatile compounds from all amounts of senescent and nonsenescent leaves, except 1 g of green leaves on radicle growth, significantly inhibited radicle, hypocotyl, and seedling growth of *Amaranthus retroflexus* (Table 5). Seed germination was drastically reduced only by 3 g of both kinds of leaf residues. However, no clear trend of differences was found in all growth parameters tested in relation to different amounts of senescent and nonsenescent leaves.

## DISCUSSION

The reduced growth of test species under sour orange trees was apparently not due to competition because there were no significant differences in pH, soil moisture, organic carbon, and amounts of mineral elements measured under sour orange and date palm trees.

TABLE 3. EFFECTS OF SOUR ORANGE LEAF EXTRACTS ON SEED GERMINATION AND SEEDLING GROWTH OF TEST SPECIES

| Species | Treatments | Oven-dry weight (mg)[a] | | | Germination |
|---|---|---|---|---|---|
| | Leaf extracts | Root | Top | Whole plant | (% of control) |
| *Avena Sativa* | Control | 131.2 | 90.2 | 221.4 | 100.0 |
| | Senescent | 30.7[b] | 67.6[b] | 98.3[b] | 29.5 |
| | Nonsenescent | 77.2[b] | 88.1 | 165.3[b] | 34.3 |
| *Amaranthus retroflexus* | Control | 25.1 | 76.4 | 101.5 | 100.0 |
| | Senescent | 10.8[b] | 30.9[b] | 41.7[b] | 85.0 |
| | Nonsenescent | 8.7[b] | 20.8[b] | 29.5[b] | 100.0 |
| *Cynodon dactylon* | Control | 18.0 | 36.3 | 54.3 | 100.0 |
| | Senescent | 8.0[b] | 10.2[b] | 18.2[b] | 24.0 |
| | Nonsenescent | 11.1[b] | 12.6[b] | 23.7[b] | 43.3 |
| *Chenopodium album* | Control | 22.3 | 34.3 | 56.6 | 100.0 |
| | Senescent | 11.9[b] | 14.7[b] | 26.6[b] | 14.5 |
| | Nonsenescent | 3.3[b] | 7.2[b] | 10.5[b] | 31.3 |

[a] Average of at least 15 seedlings.
[b] Dry weight of test significantly different from control at 0.05 level.

TABLE 4. EFFECTS OF SOUR ORANGE DECAYING SENESCENT AND NONSENESCENT LEAVES ON SEED GERMINATION AND SEEDLING GROWTH OF TEST SPECIES

| | Treatments | Oven-dry weight (mg)[a] | | | |
|---|---|---|---|---|---|
| Species | Leaf residues | Root | Top | Whole plant | Germination (% of control) |
| *Avena sativa* | Control | 41.0 | 140.0 | 181.0 | 100.0 |
| | Senescent | 44.5 | 70.1[b] | 114.6[b] | 107.8 |
| | Nonsenescent | 31.0[b] | 129.0[b] | 160.0[b] | 15.5 |
| *Amaranthus retroflexus* | Control | 15.1 | 32.1 | 47.2 | 100.0 |
| | Senescent | 7.2[b] | 16.9[b] | 24.1[b] | 72.1 |
| | Nonsenescent | 10.3[b] | 34.8 | 45.1 | 69.0 |
| *Cynodon dactylon* | Control | 11.3 | 10.0 | 21.3 | 100.0 |
| | Senescent | 5.3[b] | 8.9 | 14.2[b] | 40.8 |
| | Nonsenescent | 4.0[b] | 4.1[b] | 8.1[b] | 31.4 |
| *Chenopodium album* | Control | 26.0 | 88.4 | 114.4 | 100.0 |
| | Senescent | 26.0 | 71.8[b] | 97.8[b] | 15.5 |
| | Nonsenescent | 12.0[b] | 76.7[b] | 88.7[b] | 15.5 |

[a] Average of at least 18 seedlings.
[b] Dry weight of test significantly different from control at 0.05 level.

TABLE 5. EFFECTS OF VOLATILE COMPOUNDS FROM SOUR ORANGE SENESCENT AND NONSENESCENT LEAVES ON SEED GERMINATION AND SEEDLING GROWTH OF *Amaranthus retroflexus*[a]

| | Nonsenescent leaves | | | | Senescent leaves | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean length (mm) | | | | Mean length (mm) | | | |
| Amount of residue (g) | Radicle | Hypocotyl | Seedling | Seed germination (% of control) | Radicle | Hypocotyl | Seedling | Germination (% of control) |
| Control | 14.9a | 14.6a | 29.5a | 100 | 14.9a | 14.6a | 29.5a | 100 |
| 0.5 | 9.7b | 10.7b | 20.4b | 96 | 6.9b | 6.9b | 13.8b | 92 |
| 1.0 | 13.8a | 9.3b | 23.1b | 92 | 7.6b | 5.5c | 13.1b | 96 |
| 3.0 | 4.9c | 4.4c | 9.3c | 68 | 5.9c | 5.8c | 11.7c | 52 |

[a]Values in each column followed by the same letters are not significantly different at 0.05 level according to Duncan's multiple-range test.

Soil under sour orange trees significantly reduced seed germination and/or seedling growth of test species. This is an indication that soil under sour orange contains allelopathic compounds.

Aqueous extract of sour orange senescent leaves was found to be as inhibitory to the test species as an extract of nonsenescent leaves. This result is very striking from an ecological standpoint, since the senescent leaves represent the main source of litter under field conditions. Phytotoxic compounds in living leaves may also have an important role in inhibiting the associated species, since these compounds can be released into the environment through leaching during the rain (Al-Naib and Rice, 1971; Rice, 1974, 1979).

The results clearly showed inhibitory action of volatile compounds released from nonsenescent and senescent leaves. This result is particularly important since the liberation of volatile inhibitors is more common in plant species in the arid and semiarid zones such as the study area (Del-Moral and Gates, 1971; Horsley, 1977).

It is clear from the previous experiments that allelopathy was the basic phenomenon responsible for the failure of test species to grow normally under sour orange trees. However, once seed germination and growth of the test species are inhibited by phytotoxic compounds, competition undoubtly accentuates the growth inhibition.

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# ALLELOPATHIC EFFECTS OF *Citrus aurantium* L. II. Isolation, Characterization, and Biological Activities of Phytotoxins

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**Abstract**—In an earlier work, we found that the failure of herbaceous plants to grow under sour orange was mainly due to an allelopathic mechanism. Four nonvolatile inhibitors were isolated by paper chromatography. Color reactions of all the inhibitors indicated that they are phenolic in nature. On the other hand, five volatile inhibitors were detected by gas chromatography, four of which were terpenes. All inhibitors reduced seed germination and/or seedling growth of *Amaranthus retroflexus*.

**Key Words**—*Citrus aurantium*, allelopathy, phenols, terpenes, inhibitors, paper chromatography, gas chromatography, *Amaranthus retroflexus*.

## INTRODUCTION

The allelopathic effects of numerous kinds of chemical compounds released from plants into the environment have been discussed by many investigators (Tukey, 1969; Harborne, 1977; Horsley, 1977; Rice, 1979). The ecological importance of these compounds in natural and manipulated ecosystems has attracted the attention of many scientists. Whittaker (1971) and recently Rice (1979) summarized the role of the allelopathic compounds in plant succession, structure of plant community, dominance, diversity, vegetational patterning, and plant productivity.

In an earlier investigation (AlSaadawi and AlRubeaa, 1985), it was found that the failure of herbaceous species to grow under *Citrus aurantium* was mainly due to an allelopathic mechanism. Moreover, volatile and nonvolatile compounds were suggested to be reponsible for the reduction in growth of test species.



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However, the nature and characteristics of the inhibitory compounds responsible for the inhibition were not investigated. Therefore the present study was conducted to isolate, characterize, and/or identify the allelopathic compounds present in *Citrus* leaves and determine their biological activities.

## METHODS AND MATERIALS

*Isolation and Characterization of Inhibitors.* The procedure used to isolate and characterize the nonvolatile inhibitors was the same as described by Al-Saadawi and Rice (1983). Twenty grams from senescent and nonsenescent sour orange leaves were boiled separately in 200 ml distilled water for 10 min, ground in an electrical grinder for 10 min, and allowed to stand for 30 min. The extracts were filtered, acidified to pH 2 with N HCl, and extracted three times with half volumes of diethyl ether. Ether and water fractions were evaporated to dryness and taken up in 15 ml of absolute ethanol and 15 ml of methanol, respectively.

One milliliter from each fraction was streaked on Whatman No. 3 MM chromatographic paper, developed in butanol–acetic acid–water (BAW, 63 : 10 : 27 v/v), and examined under short and long UV with and without ammonia. All fluorescent bands were cut out and eluted with 60% aqueous ethanol. The eluates were streaked on Whatman No. 3 MM paper and developed in 6% aqueous acetic acid (6% AA). The compounds were marked under UV light, cut out, and eluted with 50% ethanol. Each eluate was chromatographed in four different solvent systems: BAW, 6%, AA, isopropanol–ammonia–water (IBW, 200 : 10 : 20 v/v), and isopropanol–*n*-butanol–water (IBW, 70 : 10 : 20 v/v). The $R_f$ values in various solvent systems, colors under UV light, colors in various reagents, and maximum absorption peaks in 50% ethanol were determined for all allelopathic compounds.

Gas chromatography was used for identification of volatile inhibitors in sour orange leaves. Thirty grams of macerated senescent and nonsenescent leaves were placed in 500-ml cylinders separately. The cylinders were tightly sealed and left for 1 hr under laboratory conditions. Ten milliliters from the atmosphere of each cylinder were taken by syringe and injected immediately into a Packard 419 gas–liquid chromatograph fitted with dual FID with the following conditions. The analytical column was 210 × 0.2 cm OD, packed with 5% SE-30 on 100–120 mesh Diatomite C-AW. The temperature programming was set from 80°C to 180°C at an increasing rate of 4°C/min. The flow rates of the gases were: N, 30 ml/min; H, 30 ml/min; and air, 200 ml/min. The oven temperature was 260°C and injection temperature was 260°C. The chart speed was 1 cm/min. The attenuation was set depending on the concentration of the sample, usually at 256 × 10.

Standards of several volatile compounds (Fluka Company) were injected under the same experimental conditions and used for identification by spiking the samples of test compounds.

*Biological Activities of Isolated Phytotoxins.* The biological activity of allelopathic compounds was determined using *Amaranthus retroflexus* seeds, one of the test species used in previous study (AlSaadawi and AlRubeaa, 1985). All destinctive bands resulting from the previous experiment were cut out, eluted, with 50% ethanol, evaporated to dryness (under vacuum), and the residues dissolved in 6 ml distilled water. Chromatography paper developed in the same solvent systems (BAW and 6% AA) without application of the extracts was subjected to the same procedure and used as a control. The aqueous solution of each band was added to a 5-cm Petri dish containing 20 $cm^3$ washed quartz sand and 25 seeds of *A. retroflexus*. The Petri dishes were placed in a growth chamber with a 14-hr photoperiod (1500 ft-c) and 26 $\pm$ 2°C/24 $\pm$ 2°C light–dark conditions. Seed germination and radicle and hypocotyl lengths were measured eight days after planting.

The biological activities of the volatile compounds isolated from sour orange leaves were determined using the general method outlined by AlSaadawi and AlRubeaa (1985). Fifty seeds of *A. retroflexus* were planted in a 9-cm Petri dish containing 40 $cm^3$ washed sand and 13 ml distilled water. Each Petri dish was placed on the perforated porcelain plate of the desiccator (2500 $cm^3$ size). Ten and twenty lambda of each of the identified terpenes and 2.5 $\mu$l and 5 $\mu$l of octanol were placed separately in the base of the desiccator; therefore, only atmospheric contact existed between the test seeds and the volatile compounds present. The control was run similarly except the volatile compounds were omitted. The desiccators were sealed tightly and kept in a growth room programmed for a 14-hr photoperiod (1500ft-c), and 26 $\pm$ 2°C/24 $\pm$ 2°C day–night conditions. Seed germination and radicle and hypocotyl lengths were measured eight days after planting.

## RESULTS

*Isolation and Characterization of Inhibitors.* Four inhibitors were isolated by paper chromatography, and they were found in both senescent and nonsenescent sour orange leaves (Table 1). All inhibitors were found in appreciable amounts in the water fraction. We were not successful in identifying the compounds through comparison of $R_f$ values, color reagents, and absorption spectra with several known inhibitors available in our laboratory. However, all inhibitors gave positive colors with most of the chemical reagents, suggesting their phenolic characteristics.

Nine major compounds were isolated by gas chromatography of the volatile compounds of macerated senescent and nonsenescent sour orange leaves (Figure 1). Based on the retention times of the available standards used, $\alpha$-pinene, $\beta$-pinene, ($\pm$)-limonene, octanol and citronellal were identified among the nine compounds isolated.

*Biological Activity of Inhibitors.* All the compounds isolated by paper chromatography significantly reduced radicle growth of *A. retroflexus* (Table 2). Ep-

TABLE 1. CHROMATOGRAPHY OF PHYTOTOXINS EXTRACTED FROM SENESCENT AND NONSENESCENT SOUR ORANGE LEAVES

| | $R_f$ on Whatman No. 3[a] | | | | Fluorescence | | Reagent colors[b] | | | Maximum absorption in 50% methanol |
|---|---|---|---|---|---|---|---|---|---|---|
| Compound | BAW | 6% AA | IBW | IAW | Long UV | Short UV | *p*-Nitroaniline | Sulfanilic acid | $FeCl_3^-$-$K_3Fe(CN)_6$ | (μm) |
| A | 79.2 | 70.5 | 72.5 | 77.6 | blue | blue | brown | yellow | blue | 279, 233 |
| B | 47.2 | 85.4 | 25.3 | 10.6 | blue | blue | brown | yellow | blue | 235, 277 |
| C | 61.2 | 65.5 | 69.8 | 81.0 | blue | blue | brown | yellow | blue | 237 |
| D | 49.1 | 27.2 | 58.5 | 59.0 | absorption | absorption | brown | yellow | blue | 238 |

[a] See text for solvent system. $R_f$ are average of three runs.
[b] Diazotized *p*-nitroaniline (Bray et al., 1950), diazotized sulfanilic acid (Bray et al., 1950), ferric chloride-potassium ferricyanide (Smith, 1960, p. 324).

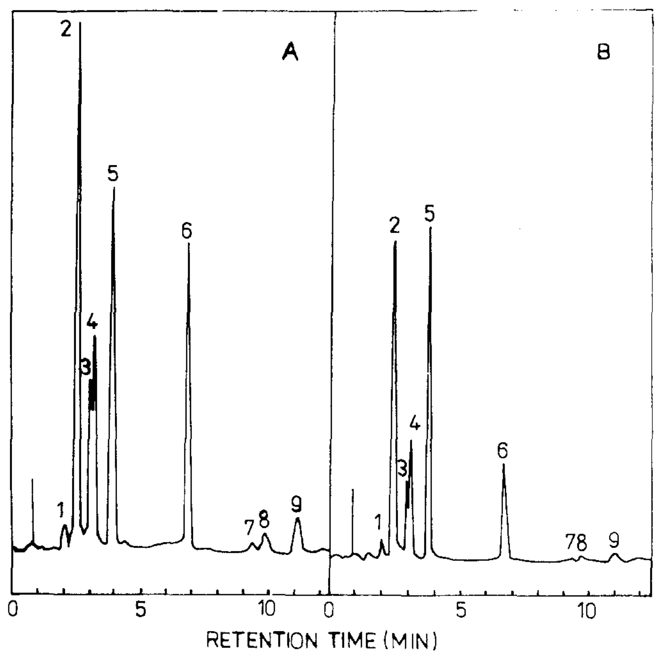


FIG. 1. Gas chromatogram of atmosphere containing macerated sour orange leaves (A) Nonsenescent leaves; (B) senescent leaves. The numbered peaks correspond to: (1) α-pinene, (2) β-pinene, (3) (±)-limonene, (4) octanol, (5) citronellal.

TABLE 2. EFFECTS OF INHIBITORY COMPOUNDS FROM SOUR ORANGE SENESCENT AND NONSENESCENT LEAVES ON SEED GERMINATION AND SEEDLING GROWTH OF *Amaranthus retroflexus*

| Compound | Mean length (mm)[a] | | | Germination (% of control) |
|---|---|---|---|---|
| | Radicle | Hypocotyl | Seedling | |
| Control | 22.1 | 11.7 | 33.8 | 100 |
| A | 11.5[b] | 8.6[b] | 20.1[b] | 100 |
| B | 18.2[b] | 11.5 | 29.7 | 100 |
| C | 11.8[b] | 8.7[b] | 20.5[b] | 81.8 |
| D | 17.7[b] | 11.4 | 29.1[b] | 77.4 |

[a] Average of at least 30 seedlings.
[b] Mean lengths significantly different from control at 0.05 level.

icotyl growth was significantly inhibited by compounds A and C only; on the other hand, seedling growth was significantly reduced by all compounds except B. Compounds C and D appreciably reduced seed germination. Compounds A and B had no effect on germination.

Radicle growth was found to be significantly inhibited by all concentrations of the volatile compounds tested, except 10 μl of (+)-limonene (Table 3). Concentrations of all test compounds significantly reduced hypocotyl and seedling growth of *A. retroflexus*. In all tests, the amount or reduction increased with an increase in amount of volatile compounds. Octanol and β-pinene were the most toxic compounds.

Seed germination was completely inhibited by the 20-μl and 5-μl concentrations of β-pinene and octanol, respectively. Two and a half microliters of octanol and 10 μl of all the remaining compounds except (−)-limonene appreciably inhibited the germination of *Amaranthus retroflexus* seeds.

Seed germination was drastically reduced by the 20-μl concentration of citronellal; however, the same concentration of all the remaining compounds, except β-pinene, did not show appreciable inhibitory effects on germination of seeds.

## DISCUSSION

Previous study revealed that the clear reduction in growth of herbaceous plants under citrus was mainly due to volatile and nonvolatile inhibitors in senescent and nonsenescent sour orange leaves (AlSaadawi and AlRubeaa, 1985). The present investigation revealed that the same volatile and nonvolatile bioactive compounds were present in senescent and nonsenescent sour orange leaves.

TABLE 3. GERMINATION AND GROWTH OF *Amaranthus retroflexus* IN ATMOSPHERE CONTAINING VOLATILE COMPOUNDS ISOLATED FROM SOUR ORANGE LEAVES

| Compound | Amount ($\mu$l) | Mean length (% of control)[a] | | | Seed germination (% of control) |
|---|---|---|---|---|---|
| | | Radicle | Hypocotyl | Seedling | |
| $\alpha$-Pinene | 10 | 52.53 | 49.07 | 50.79 | 87.5 |
| | 20 | 21.21 | 34.25 | 27.73 | 95.8 |
| $\beta$-Pinene | 10 | 31.22 | 42.84 | 37.01 | 67.5 |
| | 20 | | | | 0.0 |
| (+)-Limonene | 10 | 95.35[b] | 79.20 | 87.28 | 87.5 |
| | 20 | 47.90 | 35.28 | 41.58 | 95.8 |
| (−)-Limonene | 10 | 68.51 | 31.17 | 49.80 | 100.0 |
| | 20 | 42.92 | 32.54 | 37.72 | 93.5 |
| Octanol | 2.5 | 24.71 | 30.95 | 27.83 | 87.5 |
| | 5 | | | | 0.0 |
| Citronellal | 10 | 86.17 | 68.84 | 77.50 | 66.6 |
| | 20 | 64.69 | 36.71 | 50.70 | 13.3 |

[a] Average of at least 30 seedlings.
[b] Mean lengths were not significantly different from control at 0.05 level.

Moreover, all four nonvolatile inhibitors, which were found to be phenolic in nature, were apparentely significant from an ecological standpoint since these compounds were water soluble and could be easily leached out of the leaves by rain while the leaves are still attached to the plant or from dead leaves once the membranes are no longer functioning. The distribution of phenolic compounds in plants and their allelopathic effects in natural and manipulated ecosystems were discussed by Rice (1979) and recently by Putnam (1983).

Our results clearly indicated that all identified volatile compounds were inhibitory to seed germination and/or seedling growth of *Amaranthus retroflexus*. The result is particularly important from an ecological point of view since the liberation of volatile inhibitors is more common in plant species in a semiarid zone such as the study area (Del Mora and Gates, 1971). The inhibitory effects of volatile compounds were investigated by many scientists. Muller (1966) indicated that terpenes can be adsorbed onto soil particles during the dry summer and inhibit herb seedlings from growing the next year. Del Moral and Muller (1970) found that some volatile inhibitors produced by *Eucalyptus camaldulensis* were very important in the allelopathic activity of this species, since they were found to be adsorbed in the field in significant amounts.

It is difficult to evaluate the cumulative effects of the volatile and nonvolatile inhibitors isolated. However, the additive effect of a combination of these inhibitors is undoubtedly more detrimental than each compound separately (Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978).

The evidence is thus strong that sour orange leaves contain volatile and nonvolatile inhibitors that reduce germination and growth of test species. This allelopathic potential is considered an important component of the interference exerted by sour orange against some weedy species. Moreover, our report is the first one implicating octanol and citronellal in allelopathic activities of higher plants.

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# USE OF DYE-LABELED PROTEIN AS SPECTROPHOTOMETRIC ASSAY FOR PROTEIN PRECIPITANTS SUCH AS TANNIN

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**Abstract**—Bovine serum albumin has been covalently labeled with Remazol brilliant blue R to provide a substrate for a convenient spectrophotometric assay for protein precipitants. The blue protein is especially useful for measuring protein precipitation by vegetable tannins because its absorption maximum is at a wavelength where plant pigments exhibit minimum absorption. Blue BSA has been used to determine, by competition experiments, the relative affinity of various proteins for tannins. A procedure for purifying condensed tannin from commercially available quebracho extract is described.

**Key Words**—Tannin assay, protein precipitation, sorghum tannin, quebracho tannin, condensed tannins.

## INTRODUCTION

The biological effects of the complex polyphenols known as tannins are considered to be the result of their binding and precipitating proteins (McManus et al., 1981). Assays of protein binding and precipitation are therefore of considerable importance in characterizing tannins.

Unfortunately, the assays presently available for measuring protein binding/precipitation are not satisfactory in all respects. The use of hemoglobin as a spectrophotometric marker for protein precipitation (Bate-Smith, 1973; Schultz et al., 1981) is absolutely dependent on freshly prepared hemoglobin (I. Baldwin, personal communication); commercial (lyophilized) preparations are unsatisfactory. Pigments such as anthocyanidins, often present in plant extracts containing tannins, absorb at similar wavelengths as hemoglobin, interfering with the precipitation assay and causing high blank values. The hemoglobin



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precipitation assay has given unsatisfactory results on high-tannin sorghum (Bullard et al., 1981).

Martin and Martin (1983) devised an indirect assay using the Bradford protein test (Bradford, 1976) to measure unprecipitated protein. This technique is versatile with respect to assay conditions and has recently been adapted to the analysis of multiple samples (Wilson, 1984). It is inherently less accurate because it does not measure the precipitated protein directly. Moreover, controls to eliminate the effect of interfering materials are laborious.

There have been several attempts to estimate tannin concentration by its inhibition of various enzymes (Davis and Hoseney, 1979; Becker and Martin, 1982), but the correlation between tannin concentration and degree of inhibition is unsatisfactory (Daiber, 1975; Gupta and Haslam, 1980; Earp et al., 1981; Bullard et al., 1981). This may be due to retention of variable activity in enzyme–tannin complexes (Armstrong, 1983; Butler et al., 1984).

In this laboratory we have directly measured protein binding and precipitation by utilizing standard proteins labeled with radioisotopes in order to facilitate their detection (Hagerman and Butler, 1980a; Asquith et al., 1983). This method is sensitive and reliable, but it depends upon the availability of radioisotope equipment, and preparation of labeled protein may be difficult.

We report here a direct spectrophotometric assay which obviates most of the difficulties mentioned above. The assay utilizes a standard soluble protein, bovine serum albumin (BSA), covalently labeled with a blue dye. The assay can be adapted to measurement of materials which do not precipitate proteins but which compete with precipitants for binding them.

## METHODS AND MATERIALS

All chemicals were reagent grade and used without further purification. Bovine serum albumin (fraction V, fatty acid free), chicken egg ovalbumin, and fetuin were purchased from Sigma Chemical Corp. (St. Louis, Missouri). Calf skin gelatin was from Eastman Organics (Rochester, New York). Rat submaxillary gland glycoprotein $GP_{66}$-SMX (Mehansho and Carlson, 1983) was generously provided by Dr. Haile Mehansho. Remazol brilliant blue R was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Cyanidin was purchased from K & K Laboratories Inc. (Plainview, New York). Condensed tannin (Gupta and Haslam, 1980) was purified from *Sorghum bicolor* Moench, DeKalb BR 64, as described by Hagerman and Butler (1980b). Crude quebracho condensed tannin (Roux, 1957) was obtained from Trask Chem. Corp. (Marietta, Georgia).

*Preparation of Labeled BSA.* The protein-labeling procedure was adapted from Rinderknecht et al. (1968). To 2 g of BSA dissolved in 40 ml of 1% (w/v) $NaHCO_3$, pH 8.2, was added 150 mg of Remazol brilliant blue R, and

the solution was stirred for 30 min at room temperature. The solution was dialyzed against 0.2 M acetate, pH 4.8 (solution A), at 4°C overnight to replace the reaction buffer with a buffer more suitable for protein-binding assays. Protein concentration was determined by the method of Lowry et al. (1951). When the labeled protein had been diluted to about 1 mg/ml, the dye did not interfere with the Lowry assay.

*Protein Precipitation.* To 4.0 ml of blue BSA (2.0 mg/ml) in solution A was added 1.0 ml of methanol containing 0.1–0.7 mg of tannin [either purified tannin or a crude methanol or acidic methanol extract of plant tissue (Asquith et al., 1983)]. Five minutes after vigorous mixing at room temperature, the tubes were centrifuged (bench top centrifuge). The supernatant layer was removed with a Pasteur pipet and discarded. Precipitates were dissolved in 3.5 ml of 1% (w/v) sodium dodecyl sulfate–5% (v/v) triethanolamine–20% (v/v) isopropanol (solution B), and the absorbance at 590 nm was measured spectrophotometrically. Complete precipitation gave $A_{590}$ values of about 0.90. For blanks, methanol was substituted for the sample. Blank values are zero for properly prepared protein; no precipitate forms. Sorghum grain samples were extracted initially with hexane to remove lipids that otherwise precipitate out of the methanol extract in the aqueous assay. The assay was standardized with purified sorghum tannin. Assays of tannins from other sources should be standardized with purified tannin from that source.

*Competition Assays.* Conditions were adapted from Hagerman and Butler (1981). Varying amounts of competitor were mixed with 1 mg of blue BSA to give a total volume of 1.6 ml of solution A. To this mixture was added 0.4 ml of methanol containing enough tannin to precipitate 70–80% of the blue protein as determined in the absence of competitor. After vortexing and centrifuging as described above, the supernatant layer were removed. The pellet was dissolved in 3.5 ml of solution B and the absorbance was measured at 590 nm. For comparing the tannin-binding capacities of several proteins, it is useful to determine the concentration of competitor which inhibits the precipitation of labeled BSA in the standard assay by 50%. Relative affinity is defined as the weight of the blue BSA present divided by the weight of competitor which prevents 50% of the labeled BSA from precipitating.

*Preparation of Quebracho Tannin.* Crude quebracho tannin (20 g) was dissolved in 1 liter of 0.001 M acetic acid and extracted four times with equal volumes of ethyl acetate. Residual ethyl acetate was removed by rotary evaporation and the aqueous tannin solution mixed with a thick aqueous slurry (1 ml of resin/mg of tannin) of Sephadex LH-20 (Pharmacia). After stirring for 2 min, the slurry was transferred to a sintered glass funnel and washed successively with water and ethanol. Tannin was eluted with acetone–water (50:50, v/v). Acetone was removed from the tannin solution under reduced pressure, and the material was lyophilized.

Lyophilized tannin was further purified by applying 50 mg of tannin, in 10 ml of water, to a 2 × 15-cm column of Sephadex LH-20 equilibrated with water. The loaded column was washed with four column volumes each of water, 5% acetone (v/v), and 30% acetone (v/v). Tannin was eluted with 60% acetone (v/v) and lyophilized after the acetone was removed by rotary evaporation.

Purified quebracho tannin contained 1.3% contaminating protein as determined by Kjeldahl analysis; comparable preparations of sorghum tannin contained similar amounts of protein (Hagerman and Butler, 1978). On HPLC most of the $A_{280}$ absorbing material eluted at retention times characteristic of high-molecular-weight procyanidin polymers (Putman and Butler, unpublished data). Digestion of the tannin by HCl–*n*-butanol (Gupta and Haslam, 1980) and analysis by TLC using Analab silica gel plates and toluene–formic acid–acetone (60 : 30 : 10) (Armstrong, 1983) gave cyanidin and another compound with characteristics consistent with that of fisetinidin (Roux, 1957). Sorghum tannin yields only cyanidin when digested (Strumayer and Malin, 1975). The average chain length (Butler et al., 1982) of the purified quebracho tannin was about 70% of the chain length determined for purified sorghum tannin.

## RESULTS

*Characteristics of Blue BSA.* Blue BSA, prepared as described, can be stored for several months at 6°C, at concentrations up to 50 mg/ml, with no loss of precipitating activity. It is soluble in solution B, and the color formed is stable indefinitely. Blue BSA dissolved in solution B has an $A_{max}$ at 590 nm and a second peak at 620 nm (Figure 1). When subjected to SDS gel electrophoresis (Laemeli, 1970), the labeled protein yielded a single major blue band with an $R_f$ slightly greater than that of unlabeled BSA. Only a trace of unlabeled BSA was detectable by silver staining of these gels (BioRad, 1982).

After lyophilization, blue BSA is less soluble. Increasing the pH of the labeling reaction increases the amount of dye bound (Daytner and Finnemore, 1973), but the more heavily dyed protein is less soluble. In both cases, addition of methanol without tannin results in precipitation of blue BSA. The conditions described here, 1 mg of dye to 13 mg of BSA, represent a satisfactory compromise between color intensity and solubility properties similar to those of the native protein. Although batch to batch variation was small, each batch should be restandardized with purified tannin (see below).

*Direct Precipitation Assay.* When excess blue BSA is present, the amount of protein precipitated is proportional to the amount of added tannin (Figure 2), with sorghum tannin precipitating about twice as much blue BSA as does the same amount of quebracho tannin. Extracts from oak leaves were also assayed satisfactorily using the procedure (data not shown). The assay is sensitive to the nature of the sample solvent. Both of the purified tannins tested precipitate more

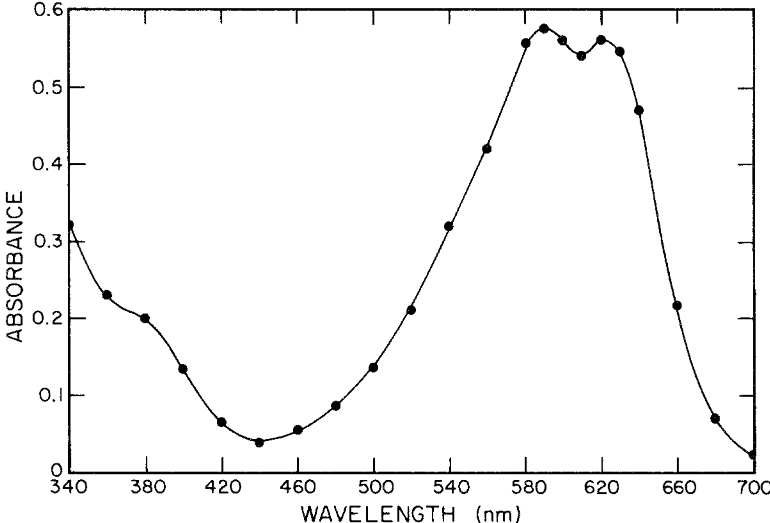


Fig. 1. Spectrum of blue BSA dissolved in solution B (0.6 mg/ml).

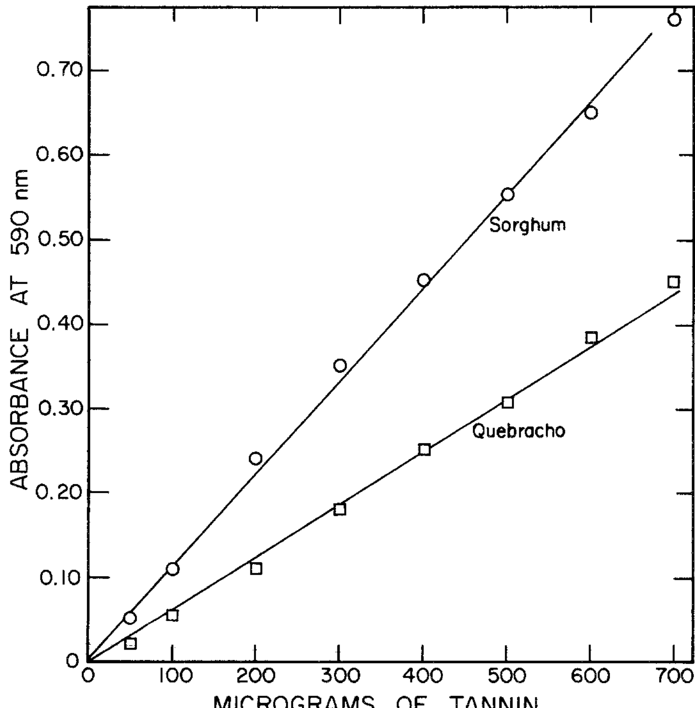


Fig. 2. Precipitation of blue BSA by sorghum or quebracho tannin under the standard assay conditions outlined in Methods and Materials. Values presented are average of duplicates.

protein when the solvent contains 20% (v/v) alcohol than in 100% water; methanol gives more precipitation than ethanol. A similar effect was observed with $^{125}$I-labeled BSA (Hagerman and Butler, 1980a). Calderon et al. (1968) noted that ethanol increased the amount of gelatin precipitated by quebracho tannin, possibly by decreasing the solubility of the tannin/protein complex.

Increasing the ionic strength decreases the amount of blue BSA precipitated (data not shown). Below pH 4 and above pH 5 very little blue BSA precipitates, in accordance with the observations of Hagerman and Butler (1978) on unlabeled BSA.

When acidic methanol is used as an extractant for tannins (Asquith et al., 1983), a few low-tannin sorghums yield a hydrophobic protein, mol wt 16,000 on SDS-PAGE (Laemeli, 1970), which coprecipitates with blue BSA in the assay to give a cloudy white haze with $A_{590}$ up to 0.4. The hazy material interferes with making accurate spectrophotometric measurements and gives a false-positive test for tannins by precipitation of blue BSA. Precipitates formed from extracts of these lines tested negative for protein precipitable phenols (Hagerman and Butler, 1978).

*Competitive Binding Assay.* A technique similar to that of competitive antibody binding assays has been utilized by Hagerman and Butler (1981) to measure the relative affinity of various proteins and other ligands for tannin. A standard labeled protein is mixed with enough tannin to precipitate 70–80% of the labeled protein. Relative affinities are established by mixing an unlabeled competitor with the labeled protein before tannin is added. Less labeled protein is precipitated in the presence of competitor than is precipitated in the absence of competitor. The effect of the competitor is a function of its concentration, its affinity for tannin, and its capacity for binding tannin. The competitor need not precipitate the tannin, but only bind it and thus prevent it from precipitating the labeled protein.

Using this assay, the set of proteins tested were found to vary by as much as three orders of magnitude in their relative affinities for the tannin (Figure 3). Results are presented as semilog plots in order to accommodate the wide range of competitor concentrations. The relative affinities of sorghum tannin for gelatin, fetuin, and unlabeled BSA were almost identical to the relative affinities of quebracho tannin for these proteins (Table 1). Compared on the basis of amount of competitor required to inhibit 50% of the precipitation due to tannin, fetuin is 10 times more effective than unlabeled BSA at binding tannin, and gelatin is about three times more effective than fetuin. Unlike other proteins, $GP_{66}$-SMX differs in its affinity for the two tannins. Soluble polyvinylpyrrolidone (PVP), a nonprotein synthetic polymer used for binding tannins in plant extracts (Gray, 1978), bound tannin in this assay with an affinity similar to that of gelatin (data not shown).

Both direct precipitation and competitive binding assays have been done

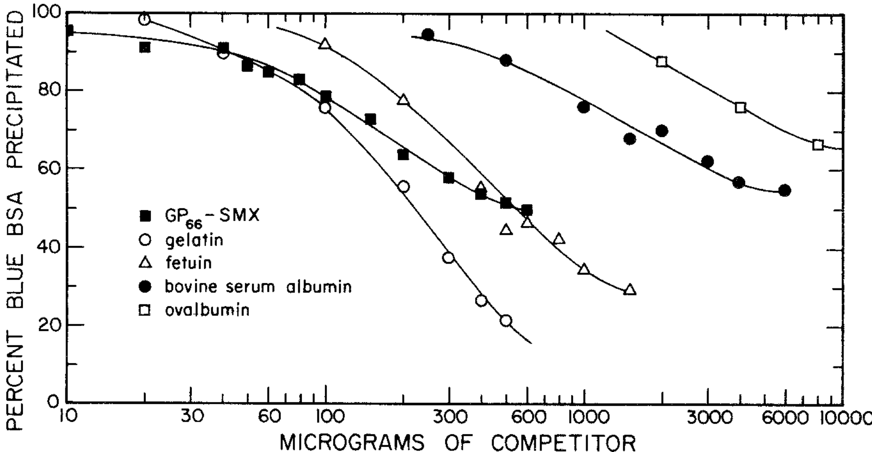


Fig. 3. Competition assays between blue BSA and standard proteins for sorghum tannin. The assays were performed as described in Methods and Materials, using 0.2 mg of purified tannin dissolved in 0.5 ml of methanol. Values presented are average of duplicates.

with tannic acid, with results similar to those obtained for the condensed tannins.

## DISCUSSION

We chose Remazol brilliant blue R as a convenient spectrophotometric label for a protein to be precipitated by tannins. It reacts irreversibly with BSA

TABLE 1. RELATIVE AFFINITY OF PROTEINS FOR TANNINS

| Protein | Relative affinity | |
|---|---|---|
| | Sorghum tannin[b] | Quebracho tannin[b] |
| Ovalbumin | 0.05 | 0.003 |
| BSA | 0.17 | 0.17 |
| Fetuin | 1.8 | 1.8 |
| Gelatin | 4.5 | 5.8 |
| $GP_{66}$-SMX | 1.7 | 12. |

[a]0.2 mg of sorghum tannin was used.
[b]0.3 mg of quebracho tannin was used.

under mild conditions without apparent denaturation. Its maximum absorption is at longer wavelengths than the anthocyanidins and other plant pigments often associated with tannins, thus minimizing their interference with the assay. Introduction of the hydrophobic dye reduces the solubility of the BSA and makes it precipitate more readily than unlabeled BSA.

This assay should be applicable to determination of tannin in a wide variety of plants. It must be noted, however, that direct precipitation assays, including Bate-Smith's hemanalysis (1973) and this assay with blue BSA, detect all protein precipitants. Formation of a precipitate should not be interpreted as indicative of the presence of tannin unless corroborated by assays for protein-precipitable phenols (Hagerman and Butler, 1978). It is possible that blue BSA will be useful in measuring other protein precipitants in addition to tannins.

Competition assays do not measure the concentration of tannin or any other protein precipitant. They measure the relative affinity of various materials, usually proteins, for the precipitating agent (in this case a tannin). Materials which bind tannin without precipitation are measured by this assay. The relative affinity of proteins for tannins varies widely, as previously noted (Hagerman and Butler, 1981).

The affinity of a protein for tannin may indicate functional significance (Mehansho et al., 1983). The high relative affinity for tannins of salivary proline-rich proteins such as $GP_{66}$-SMX, and their rapid induction in response to dietary tannins (Mehansho et al., 1983), suggests that these salivary tannin-binding proteins constitute a chemical defense system which binds and inactivates dietary tannins immediately on entering the digestive tract (Mehansho et al., 1983). Hamsters do not respond to dietary tannin in this manner and are relatively vulnerable to the effects of ingested tannins (Mehansho et al., 1985).

Possibly because of its higher degree of polymerization, sorghum tannin precipitates about twice as much blue BSA as an equal weight of quebracho tannin. Porter and Woodruff (1984) reported that procyanidin chain length was the determining characteristic in tannin astringency.

Tannins with different protein-precipitating capacities (Figure 2) cannot be directly compared in competition assays at the same tannin concentrations. We are preparing tannins labeled with $^{125}I$ for use in competition assays in order to compare relative affinities of sorghum, quebracho, and other tannins for standard proteins. Use of direct assays for measuring protein-precipitating capacity, and competition assays for measuring relative affinity, independent of precipitation, should increase our understanding of the specificity and mechanism of tannin binding by proteins.

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# RESPONSES OF THE AMPHIPOD CRUSTACEAN *Gammarus palustris* TO WATERBORNE SECRETIONS OF CONSPECIFICS AND CONGENERICS

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**Abstract**—A choice-test apparatus designed to mimic field conditions was employed to test for the presence of waterborne attractants in the amphipod crustacean *Gammarus palustris*. It was found that both males and females were attracted to secretions from all conspecifics, but not to the secretions of a sympatric congener. When given the choice of secretions from different types of conspecifics, males behaved differently than females. Males were attracted more often to receptive females' and females were attracted more often to males' secretions. In the field, then, it is likely that all conspecifics travel toward each other, then sort themselves into competent heterosexual couples. The results suggest that this apparatus can be employed in future studies to determine the chemical nature of these pheromones.

**Key Words**—Waterborne attractants, Crustacea, *Gammarus palustris*.

## INTRODUCTION

An increasing body of data has demonstrated the importance of waterborne pheromones in intraspecies communication in marine invertebrates. For example, they are employed to attract gametes in the hydroid *Campanularia flexuosa* (Miller and Nelson, 1962), as inducers of spawning in some species (reviewed by Mackie and Grant, 1974), and as stimuli for larval settlement in others (reviewed by Crisp, 1974). In addition, they have been implicated as mediators of courtship and pair formation in many Crustacea (see Dunham, 1978, for review), but details about the mechanism of crustacean pheromones actions are scanty (for example see Salmon, 1983). The experiments reported here were



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undertaken to supply some basic details for one species, the amphipod crustacean *Gammarus palustris*.

*Gammarus palustris* is a resident of the high-tide mark of salt marshes along the western Atlantic seaboard (R. Borowsky, 1984). It travels freely in its preferred habitat when covered by water but is quiescent under rocks and debris when exposed to the air (which occurs for about two 8-hr periods every 24 hr). Females produce several broods in succession, but require insemination before each ovulation because there is no sperm storage. Typically, a male picks up a female and carries her about until the female molts (precopulation; B. Borowsky, 1984). Immediately after the molt, copulation occurs, and the pair separates. The female then remains alone until a few days before her next molt, when precopulation is reinitiated.

In most crustaceans, the effects of females' secretions on males have been investigated, the idea being that when females are receptive they secrete substances which attract conspecific males, and/or stimulate their reproductive behaviors (Dunham, 1978). However, although individuals of both sexes are mobile, and it is as important to the receptive female to find a competent male as it is for the male to find a female, the possibility that male pheromones exist has been largely ignored. Therefore, the effects of different types of conspecifics' secretions on both males and females were investigated in *G. palustris*.

## METHODS AND MATERIALS

All animals were collected from the high-tide mark of the intertidal zone at Jamaica Bay, Gateway National Recreation Area, Brooklyn, New York. Adults were placed in one of three categories: receptive females, nonreceptive females, or males. A female was considered "receptive" if it was currently precopulating (being carried about by a male) and nonreceptive if it was both (1) not in precopulation and (2) carrying eggs at an early stage of development (eggs at the early stages contain a relatively large amount of dark brown yolk which is easily seen without the aid of a microscope). Receptive females were separated from their attached males by gently pulling the pair apart. This treatment had no apparent affect on either animal, for precopulation would immediately ensue if, after separation, they were replaced together in a dish of seawater. Subsequent survivorship was also unaffected.

The choice-test apparatus shown in Figure 1 was employed as follows: water was introduced into the apparatus in section A of each filter box at a rate of 200 ml/hr. The water then flowed into parts B of the filter boxes through a plastic mesh screen (C). The water then flowed out of each filter box through a 17.5-cm length of 1.2-cm-diameter tubing which led to one arm of a glass Y-tube (D). The streams of water from the filter boxes united at the fork of the Y and continued their flow through an additional 17.5-cm length of tubing into the

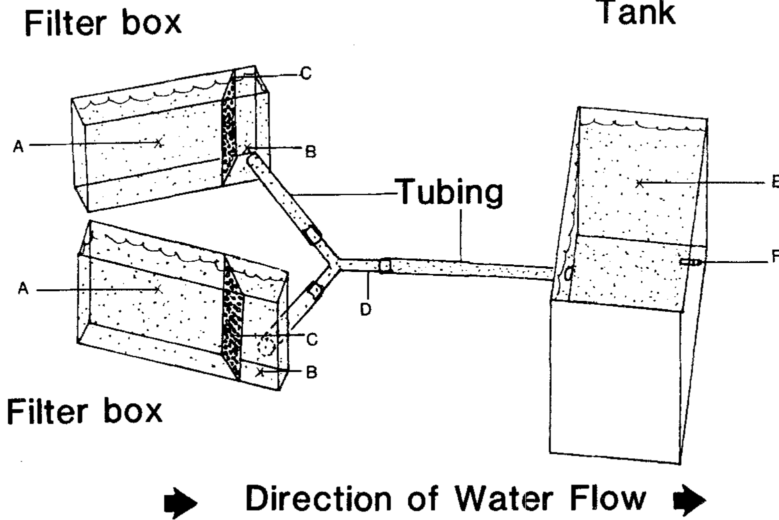


FIG. 1. Apparatus employed for testing *Gammarus palustris* individuals' responses to other animals' water-borne secretions. Animals whose secretions were tested were placed in the A sections of the filter boxes, and individuals whose responses were to be tested were placed in the Tank (E). The latter could leave the tank and travel to a Y-tube (D), where they could choose to travel to either filter box. Censuses of the number of animals in the B sections of the filter boxes revealed individuals' preferences for different types of animals' secretions. C = plastic mesh screen; F = tube through which water exited from the apparatus.

bottom of the 7-liter plastic tank (E). Water exited the system through a hole near the top of the tank (F).

Forty individuals whose secretions were to be tested were introduced into one of the filter boxes' A sections. *Ulva lactuca* thalli (the most common marine alga at the collection site) were placed in the A sections and rocks were placed in the B sections to mimic field conditions. Water was allowed to flow through the system for several hours before an experiment was conducted, to allow the concentrations of whatever secretions might be present to equilibrate in the appropriate sections of the apparatus.

A test was begun by placing 20 males or nonreceptive females in the tank. Animals could pass freely from the tank to the Y-tube and then to either of the B sections. They could also return or change direction at any time. They could not enter the A chambers, however, because of the plastic mesh partition. During an experiment, water passed over the test animals in the A section, thus carrying their secretions into the Y-tube, and finally into the tank, where they might influence the traveling animal's behavior. Twenty-four hours after the test animals were introduced, the apparatus was disassembled, and the numbers of

traveling animals in each of the B sections as well as the number in the tank were counted. Animals that were in the tubes were counted with those in the tank if they were in the stem of the Y, or with those in the left or right filter boxes if they were in the left or right arms of the Y, respectively. The apparatus was washed in hot tap water and allowed to air-dry between tests.

Four tests were conducted for each experiment for the males, but eight tests were conducted for the females because they were less active than the males. The filter box containing the animals whose secretions were being tested was placed on the left side of the apparatus for the first and third tests, and on the right side for tests two and four. First, the effects of light vs. dark on the males' responses to receptive females' secretions were tested. One experiment was conducted in the light, the other in the dark. After determining that the males were only responsive in the dark, all further tests were conducted in the dark (see Results section). Next, the males' responses to secretions from conspecific nonreceptive females, conspecific males, or receptive *Gammarus mucronatus* females were tested. *G. mucronatus* is a closely related species which is found on the same beaches as *G. palustris* (Borowsky, 1980). The next two experiments tested the relative attractiveness of the three types of conspecific adults' secretions to males in simultaneous comparisons. In these tests, 20 individuals of one type were placed in one filter box, and 20 of another type were placed in the other.

The experimental procedures employed to test the females' responses were identical to those employed to test the males', except: (1) only nonreceptive females were tested, because receptive females are typically carried about by males and remain relatively quiescent when separated from them; (2) eight, rather than four, tests were conducted for each experiment; and (3) females were exposed to secretions from competent male *G. mucronatus* rather than to receptive females of that species. Male *G. mucronatus* that were currently precopulating were classified as reproductively competent, separated from their females, and employed in these experiments.

Hypotheses were tested using the $\chi^2$ and binomial statistics. The overall significances of the experiments were obtained by combining the probabilities from separate replicates using the method of Fisher (1946). Individual probabilities for each replicate were obtained from the binomial distribution (two-tailed test) and $-2$ times the sum of their natural logarithms were taken as $\chi^2$ with eight degrees of freedom for males' and 16 for females' experiments (referred to as the "aggregate $\chi^2$").

## RESULTS

There was no significant difference between the number of males that traveled to the females' as opposed to the empty filter boxes when the tests were conducted in the light (Table 1). But males traveled significantly more often to

TABLE 1. MALES EXPOSED TO ONE TYPE OF SECRETION AT A TIME: NUMBERS OF MALES FOUND IN DIFFERENT CONTAINERS AFTER TESTS[a]

| | Filter boxes with secretions from | | | Differences in numbers of males in the two filter boxes | |
|---|---|---|---|---|---|
| | | | Tanks | Aggregate $\chi^2$ ($df$ = 8) | $P$ |
| A. In the light | | | | | |
| | Receptive females | No animals | | | |
| $\bar{X}$ (SE) | 5.7 (0.5) | 8.5 (1.0) | 5.5 (1.2) | 15.2 | > 0.05 |
| B. In the dark | | | | | |
| 1. | Receptive females | No animals | | | |
| $\bar{X}$ (SE) | 13.8 (0.6) | 4.3 (0.9) | 2.8 (0.8) | 30.9 | <0.001 |
| 2. | Nonreceptive females | No animals | | | |
| $\bar{X}$ (SE) | 10.3 (0.6) | 4.0 (1.0) | 5.3 (0.2) | 29.7 | < 0.001 |
| 3. | Males | No animals | | | |
| $\bar{X}$ (SE) | 13.3 (0.7) | 2.3 (1.1) | 4.8 (1.0) | 35.6 | < 0.001 |
| 4. | Receptive female *Gammarus mucronatus* | No animals | | | |
| $\bar{X}$ (SE) | 7.5 (0.8) | 4.5 (0.9) | 7.8 (0.8) | 10.0 | > 0.05 |

[a] $\bar{X}$ = mean, SE = standard error, $df$ = degrees of freedom.

the females' filter boxes when the tests were conducted in the dark (Table 1, B1). Thus, males were more responsive to receptive female attractants in the dark than in the light.

Males were attracted significantly often to all types of conspecifics but were not to receptive *G. mucronatus* females (Table 1, B2, 3, and 4). Thus there is a species-specific substance secreted by all types of adults which attracts males. In addition, males were attracted significantly more often to receptive females than to nonreceptive females, but they were attracted to nonreceptive females and to males about equally (Table 2, A).

Females traveled to conspecifics' filter boxes significantly more often then to empty filter boxes, but traveled about as often to competent male *G. mucronatus*' as to empty filter boxes (Table 3). Thus, female *G. palustris* are also attracted to all conspecifics. But in contrast to the males' responses to conspecifics, females traveled more often to males as opposed to other conspecifics in the stimultaneous comparison tests. There was no significant difference between the number of females that traveled to receptive vs. nonreceptive females' sides (Table 2, B1), but females traveled to males' sides significantly more often than to nonreceptive females' sides (Table 2, B2).

TABLE 2. MALES AND NONRECEPTIVE FEMALES EXPOSED TO TWO TYPES OF SECRETIONS SIMULTANEOUSLY: NUMBERS OF ANIMALS FOUND IN DIFFERENT CONTAINERS AFTER TESTS[a]

| | Filter boxes with secretions from | | Differences in numbers of animals in the two filter boxes | | |
|---|---|---|---|---|---|
| | | | Tanks | Aggregate $\chi^2$ | $P$ |
| A. Males | | | | ($df$ = 8) | |
| | 1. Receptive females | Nonreceptive females | | | |
| $\bar{X}$ (SE) | 10.8 (0.4) | 3.8 (0.8) | 5.3 (0.3) | 25.0 | < 0.01 |
| | 2. Nonreceptive females | Males | | | |
| $\bar{X}$ (SE) | 8.3 (0.7) | 8.8 (0.5) | 3.0 (0.4) | 7.16 | > 0.05 |
| B. Females | | | | ($df$ = 16) | |
| | 1. Receptive females | Nonreceptive females | | | |
| $\bar{X}$ (SE) | 5.4 (0.4) | 4.5 (0.3) | 10.1 (0.5) | 16.2 | > 0.05 |
| | 2. Nonreceptive females | Males | | | |
| $\bar{X}$ (SE) | 3.6 (0.4) | 7.1 (0.6) | 8.5 (0.6) | 30.0 | < 0.01 |

[a]$\bar{X}$ = mean, SE = standard error, $df$ = degrees of freedom.

TABLE 3. NONRECEPTIVE FEMALES EXPOSED TO ONE TYPE OF SECRETION AT A TIME: NUMBER OF FEMALES FOUND IN DIFFERENT CONTAINERS AFTER TESTS[a]

| | Filter boxes with secretions from | | Differences in numbers of females in the two filter boxes | | |
|---|---|---|---|---|---|
| | | | Tanks | Aggregate $\chi^2$ ($df$ = 16) | $P$ |
| 1. | Receptive females | No animals | | | |
| $\bar{X}$ (SE) | 7.1 (0.4) | 5.0 (0.8) | 8.3 (0.4) | 37.8 | < 0.01 |
| 2. | Nonreceptive females | No animals | | | |
| $\bar{X}$ (SE) | 10.3 (0.6) | 3.8 (0.4) | 6.9 (0.9) | 49.1 | < 0.01 |
| 3. | Males | No animals | | | |
| $\bar{X}$ (SE) | 9.0 (0.4) | 3.9 (0.4) | 7.3 (0.6) | 37.0 | < 0.01 |
| 4. | Competent male *Gammarus mucronatus* | | | | |
| $\bar{X}$ (SE) | 5.5 (0.4) | 5.5 (0.5) | 9.6 (0.5) | 13.7 | > 0.05 |

[a]$\bar{X}$ = mean, SE = standard error, $df$ = degrees of freedom.

## DISCUSSION

The results of these experiments have shed some light on the way pheromones act in *Gammarus palustris*. First, males are more responsive to attractants in the dark than in the light. This suggests that males are more active at night than during the day (an activity pattern typical of most aquatic Crustacea) (Dunham, 1983). Thus their period of activity may be very brief: confined to the 4-hr period of submersion at their high-tide mark habitat which occurs during the night. Second, a species-specific waterborne attractant to which both sexes respond exists in *G. palustris;* and third, males prefer receptive females' and females prefer males' attractants over that of other conspecifics. These observations show that there are qualitative differences between the secretions of the two sexes.

It is not surprising that *G. palustris* individuals do not respond to secretions from *G. mucronatus*. At least seven species of gammarid amphipods are permanent residents of the collection site during the warmer months (personal observation). It would be a waste of energy to travel to individuals of other species. Further, the preferred habitat of *G. palustris* is in a relatively narrow band in the intertidal zone. The secretion of a species-specific attractant may facilitate the location of adults in their habitat.

The present study reveals no information about the effects of the attractants on initiating pair formation and mating behaviors. Investigations into these questions are currently underway. However, it is highly probable that the frequencies of these behaviors are enhanced by the existence of the attractants. The data suggest that animals are more likely to travel toward, and therefore encounter, individuals of the opposite sex than individuals of the same sex, which should facilitate pair formation.

It should prove fruitful to test for male attractants in other species as well. For example, although receptive female lobsters (*Homarus americanus*) secrete a sex attractant (Atema and Engstrom, 1971), it is the females who travel to males' home sites under field conditions (Atema et al., 1979). It seems possible that the movements of the females are guided by male attractants. Finally, the results suggest that the apparatus can be employed in future studies to determine the chemical nature of the attractants.

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# USE OF CHEMICAL VARIATION AND PREDATION AS PLANT DEFENSES BY *Encelia farinosa* AGAINST A SPECIALIST HERBIVORE

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**Abstract**—Larvae of the monophagous herbivore, *Trirhabda geminata*, selectively eat particular plants and plant parts of its natural host, *Encelia farinosa*. Measurements of leaf damage and larval positions on branches through time support this observation. Time-lapse movie photography revealed that larvae are sufficiently mobile to search most of a plant in a 48-hr period and that aggregations were the result of larval activity and not directly the result of oviposition. Experiments with *T. geminata* larvae on artificial diets containing a range of natural concentrations of chemical extracts from *E. farinosa* leaves showed that the larvae grew significantly slower and had a lower overall survivorship at the high concentration. Combining the results of all choice tests, larvae appeared unable to distinguish between high- and low-concentration agar diets. Considered individually, larval preferences for natural production concentrations changed as the season progressed. Early-season larvae preferred low-concentration leaves, while late-season larvae preferred high-concentrations. Measurements of chemical and nitrogen content of leaves selected by larvae in the field confirmed this pattern. Percent parasitism in field-collected larvae increased with season as the larval population decreased. This combination of slowed growth and increasing parasitism and predation is a putative defense strategy of *Encelia farinosa* to prevent adaptation by a specialist herbivore to the total range of compounds elaborated.

**Key Words**—Herbivory, chemical quantitative variation, specialist herbivore, parasitism, growth reduction, *Encelia farinosa*, Asteraceae, *Trirhabda gem-*

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*inata*, Coleoptera, Chrysomelidae, *Chaetonodexodes vanderwulpi*, Diptera, Tachinidae.

## INTRODUCTION

Specialist insect herbivores have been characterized as being able to track their host plant spatially and temporally and to be adapted to the defensive compounds of their host (Feeny, 1976; Rosenthal et al., 1977; Kircher and Heed, 1970; Fox and Macauley, 1977; Blau et al., 1978; Isman, 1977). These studies have shown no link between either the types or amounts of natural products present in a host and the growth and fitness of the specialist herbivore. Extreme examples of adapted specialist herbivores are those insects which use the host's natural products as ovipositional cues or chemical defenses (Dethier, 1970; Schoonhoven, 1968, 1972; Harborne, 1977; Rodriguez and Levin, 1976).

However, exceptions to this general profile have been reported (Cooper-Driver and Swain, 1976; Jones, 1962; Sturgeon, 1979; Whitham, 1983; Zucker, 1982). In these studies, differential herbivory (quantitative differences in tissue loss to herbivores and herbivore distribution within and between individual plants) was observed in the interaction between specialist herbivores and their host plant. When examined, the natural product chemistry of the host plant exhibited extensive quantitative variation (Zucker, 1982). In each of these interactions, the specialist herbivores demonstrated clear differences in growth and reproduction when grown on different parts of their host plants. Herbivores clumped on high-quality plant parts are more vulnerable to predators and parasites (Whitham, 1981). Plant chemical quantitative variation may constitute a plant defense which may increase the vulnerability of the herbivores to their natural predators (Price et al., 1980; Whitham, 1981).

*Trirhabda geminata* Horn (Coleoptera: Chrysomelidae) is a monophagous leaf feeding herbivore of *Encelia farinosa* Gray (Asteraceae) which exhibits differential herbivory. Preliminary field observations of *E. farinosa* populations revealed that only certain plants and plant parts were being eaten by *T. geminata* larvae (Wisdom, personal observation). Prior chemical investigations of *E. farinosa* had documented the presence of two potential defensive chemical classes (sesquiterpene lactones and chromenes) which were highly quantitatively variable (Wisdom and Rodriguez, 1982, 1983). Additionally, extensive parasitism and predation of *T. geminata* larvae and adults occurred in these populations. This system contained all the elements of Whitham's (1981) general scenario for the defensive function of plant chemical quantitative variation. However, research on a related beetle species (also a specialist), *T. bacharidis* (Weber), with differential herbivory, showed no feeding or growth response of the larvae to seasonally increasing chemical concentrations in plant extracts (Kraft and Denno, 1982). This study was conducted to examine whether the observed chemical

quantitative variation in *Encelia farinosa* was the cause of the differential herbivory patterns of *Trirhabda geminata*.

## METHODS AND MATERIALS

All larvae of *T. geminata* and plants of *Encelia farinosa* were collected from Homes Gardens, California, 21 km west of Riverside, California, in a plant community transitional between coastal sage and Coloradan desert flora.

*Field Measurements:* The damage to leaves from *T. geminata*, the dominant herbivore, was easily distinguishable from that of other herbivores in the study sites. This leaf damage was scored for presence or absence on 2183 leaves from 197 branches of *E. farinosa* in 1980. All measurements were made after the beetles had completed their activity (larvae and adults) and before leaf abscission. Leaf area removed was not measured, as leaves had continued to expand after the damage occurred.

In the spring of 1981, the number of individual larvae on individual branches was scored on 35 haphazardly selected plants five separate times spanning a two-month period to determine the distribution pattern of larvae on a plant. The sampling unit selected was the individual branch of a single plant. Larvae appear unable to move between plants, restricting their activity to the plant on which they were oviposited (C. Wisdom, personal observation). The number of larvae per branch were counted to determine the spatial distribution of larvae within their resource base. The distribution of the number of larvae per branch on each plant was compared to the negative binomial distribution as a test of aggregation (Pielou, 1977). A computer program was used to calculate the negative binomial distribution and a goodness of fit test (see Anscombe, 1950; Evans, 1953; Bliss and Fisher, 1953; Poole, 1974; Wisdom, 1982; for further details). Two indices were used to determine if the observed larval distributions were significantly aggregated on a plant. The negative binomial parameter, $K$, generated from the observed distribution, was used as an index of the degree of aggregation. $K$ varies from zero to infinity and indicates clumping if its values are in the range of 0–8. Expected distribution of the negative binomial were generated using $K$ for goodness of fit comparisons to the observed larval distributions. Observed distributions not distinguishable from the expected distribution were presumed to be aggregated.

In order to determine the changing nature of the larval aggregations, one plant was selected for measurements of larval movement and aggregation. All the branches of this one plant were tagged and larval numbers recorded per branch on six dates over a $2\frac{1}{2}$-week period. To determine the actual pattern of daily larval movements, time lapse super-8-mm movie photography was used. Two larvae (second instar) were selected for observation. The movie camera

was set up to record the movements of each larvae on the end area of the branch. These larvae were photographed for 3-hr on a typical sunny day. Individual frames were exposed once every 5 sec for the total time period. Larval activity was scored for the patterns and rates of movement during this period.

Parasitism levels of larvae were determined by collecting third-instar larvae from *E. farinosa* plants and allowing them to reach pupation in the laboratory. Twenty-five to 35 larvae were collected on each measurement date. Collection dates started in mid-February as this coincided with the first appearance of third instars in the population. After pupation, or the development of a fly puparium, parasitized and nonparasitized larvae were counted to determine percent parasitized.

*Laboratory Measurements.* Leaves currently being eaten by *T. geminata* larvae were collected for analysis of natural product and nitrogen content. The chemical analysis methodology is the same as in Wisdom and Rodriguez (1982). Leaf percent nitrogen content was analyzed by the Agricultural Experimental Station, University of Alaska, Palmer, Alaska, using a micro-Kjeldahl digestion of samples to ammonium-nitrogen and analysis on an Autoanalyzer II using the indophenol procedure.

Feeding experiments using artificial diet were performed with first and third instars. *T. geminata* larvae would not feed on agar diets made according to the original recipe of Sutter et al. (1971) for *Diabrotica undecimpuntata howardi* (Coleoptera: Chrysomelidae). Therefore, chloroform-extracted leaves of *E. farinosa* were added at the rate of 1.00 g of leaves to 10.0 g to the Sutter diet. With this modified leaf–agar diet (containing no sesquiterpene lactones or chromenes), it was possible to raise insects from first-instar larvae to adults (no check of adult fecundity was made). A semipurified chloroform extract of *E. farinosa* leaves was added to the leaf–agar diet. This extract contained known amounts of the three principal natural products examined (farinosin, a sesquiterpene lactone, and encecalin and euparin, both chromenes) (Figure 1). Concentrations were checked by high-performance liquid chromatography. The ratio of material was approximately 80 : 15 : 15 (encecalin–farinosin–euparin). This extract was dissolved in acetone before addition to the nonsolidified diet, and the diet was allowed to harden. The acetone was allowed to evaporate until no odor was detectable. Two types of the leaf–agar diets were made containing: (1) the highest natural concentration of the combined amount of the three compounds found (high diet, 6.00% wetwt) and, (2) the lowest natural concentration found (low diet, 1.5% wetwt) (Wisdom and Rodriguez, 1982).

Two experiments were performed using the two leaf–agar diets. The first was a no-choice experiment, comparing relative growth rates (RGR) and larval survivorship on the high and low diets (30 first-instar larvae per diet, unlimited diet per larva). Larvae in the no-choice experiment were weighed at the beginning and at the end of the experiment (a three-week period). The second exper-

1) FARINOSIN    2) ENCECALIN

3) EUPARIN

FIG. 1. Structures of the three main natural products of *Encelia farinosa:* farinosin, a eudesmanolide sesquiterpene lactone, encecalin, a benzopyran chromene, and euparin, a benzofuran chromene.

iment was a choice experiment between the diet types. Weighed agar squares of the high and low diets were placed into a plastic soufflé cup (Solo Co.) with one weighed larva (first through third instars). Water loss from the squares was presumed to be equal for each square in a Solo cup. The larvae were allowed to feed for 48 hr. Both agar squares were weighed at 24 and 48 hr.

## RESULTS

The pattern of leaf damage at the end of the beetle activity season indicates that *T. geminata* larvae have a highly aggregated feeding pattern. Overall, 48% of the 2183 leaves examined were damaged. A $G$ test for heterogeneity showed the damage to be extremely heterogeneous ($G = 746.8$; $df = 44$; $P < 0.005$) (Sokal and Rohlf, 1969). Over 95% of the leaves of some branches were damaged, while other branches from the same plant had less than 10% of their leaves damaged.

Measurements of larval applications showed that larvae aggregate early in the season and that the degree of aggregation decreases as the season progresses (Figure 2). Examination of a single plant on a shorter time scale shows that clumping patterns change radically with time (Table 1). Larvae were not aggregating due to any innate gregariousness as shown by the changing composition of aggregations and the number of solitary larvae. The time-lapse movies support this observation, showing the sedentary-appearing larvae to be highly mobile.

Field collected larvae experienced a high degree of parasitism, reaching a

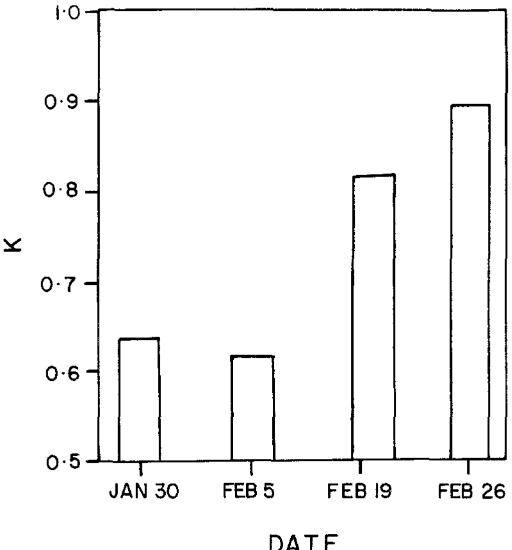


FIG. 2. Seasonal changes in the mean values of the negative binomial "clumping" parameter, $K$. $K$ ranges in value from 0 to infinity. The lower the value of $K$, the higher the degree of aggregation. The sampling unit was the number of larvae per individual branch.

high of 35% parasitized larvae in the Home Gardens Site (Figure 3). This increase in parasitism level coincided with a general drop in larval population size, indicating an asynchronous relationship between the parasite, *Chaetonodexodes vanderwulpi* (Townsend) (Diptera: Tachinidae), and its larval host, *Trirhabda geminata*.

TABLE 1. DAILY CHANGES IN NUMBERS OF LARVAE DISTRIBUTED OVER MARKED BRANCHES OF ONE PLANT[a]

| Date | 46 | 98 | 70 | 51 | 62 | 34 | 89 | 21 | Total |
|---|---|---|---|---|---|---|---|---|---|
| January 20 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| January 21 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 3 |
| January 27 | 2 | 0 | 0 | 2 | 2 | 3 | 0 | 0 | 9 |
| January 29 | 3 | 1 | 2 | 4 | 1 | 8 | 0 | 0 | 19 |
| January 30 | 3 | 4 | 2 | 3 | 0 | 6 | 0 | 0 | 19 |
| February 3 | 0 | 4 | 3 | 1 | 1 | 0 | 0 | 0 | 9 |
| February 5 | 1 | 8 | 3 | 4 | 2 | 2 | 1 | 1 | 22 |

[a]The individual larvae appear highly mobile and readily move from branch to branch. This indicates that larval aggregations are by choice and that larvae are capable of sampling the various branches in a short period of time.

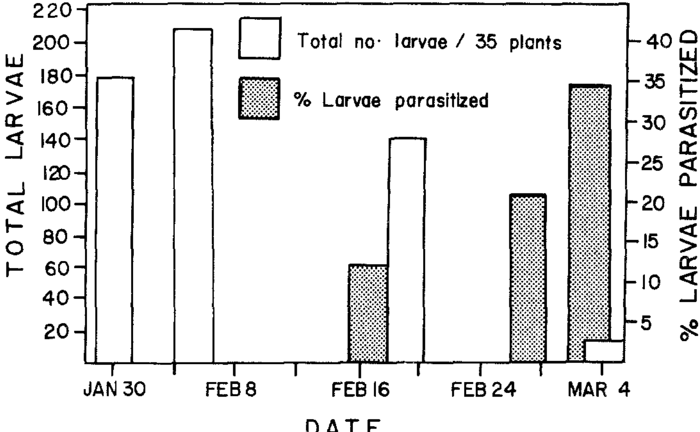


FIG. 3. The number of larvae present in Home Gardens populations of *Encelia farinosa* per 35 plants during the season of beetle activity, and the percentage of larvae parasitized from the same population is shown in this figure. Parasitism levels are calculated per approximately 30 larvae per collection date.

The growth rate and overall survivorship of first-instar larvae of *T. geminata* were significantly retarded on the high-concentration artificial diet (Tables 2 and 3). Growth rates of larvae fed the low-concentration diet were over twice that of the larvae fed high-concentration diet. Additionally, survivorship of larvae at low concentration was significantly higher than that at high concentration larvae.

The combined results of all choice experiments were insignificant. As a group, the larvae expressed no clear-cut preferences (Table 4). This experiment was run in three separate trials, one at the first of the beetle activity season, the second midseason, and the third at the end of the season. The first group, composed mostly of first instars, preferred low concentrations. The second group,

TABLE 2. MEAN RELATIVE GROWTH RATES OF FIRST-INSTAR LARVAE OF *Trirhabda geminata* ON ARTIFICIAL DIETS CONTAINING HIGH (6.00% WET WEIGHT, 30 LARVAE) AND LOW (1.5%, 30 larvae) CONCENTRATIONS OF *Encelia farinosa*.

| | Relative growth rate means after 24 days[a] | | |
|---|---|---|---|
| Concentration | Mean | Var. | $N$ (survivors) |
| Low | 0.042 | 0.00084 | 17 |
| High | 0.018 | 0.00024 | 9 |

[a] RGR = ln $(W_{final}/W_{initial})/T$ One tailed $t$ test = 2.26; $df$ = 24; $P < 0.025$.

TABLE 3. OVERALL SURVIVORSHIP RATES OF 60-FIRST-INSTAR LARVAE OF *Trirhabda geminata* ON HIGH AND LOW-CONCENTRATION DIETS

| Concentration | Survivorship after 27 days[a] | | |
|---|---|---|---|
| | Alive | Dead | N |
| Low | 10 | 20 | 30 |
| High | 3 | 27 | 30 |
| | 13 | 47 | 60 |

[a] $df = 1$, $P < 0.05$, $G = 5.023$

TABLE 4. AMOUNT OF DIET CONSUMED IN CHOICE EXPERIMENTS BY 150 LARVAE (FIRST THROUGH THIRD INSTARS) OF *T. geminata* BETWEEN HIGH- AND LOW-CONCENTRATION DIETS ON THREE SEPARATE DATES

| Exp. | Diet type | Amount of diet consumed (mg) | | *N* | *P* |
|---|---|---|---|---|---|
| | | Mean | SD | | |
| 1 | Low | 64.50 | 31.25 | 22 | <0.05 |
| | High | 45.30 | 20.00 | 22 | |
| 2 | Low | 47.50 | 23.82 | 60 | NS |
| | High | 39.67 | 34.83 | 60 | |
| 3 | Low | 43.96 | 34.05 | 68 | <0.025 |
| | High | 54.51 | 21.87 | 68 | |
| Total | Low | 48.38 | 30.51 | 150 | NS |
| | High | 47.23 | 28.25 | 150 | |

TABLE 5. CONCENTRATIONS OF FARINOSIN, ENCECALIN, EUPARIN, AND NITROGEN IN LEAVES FED UPON BY LARVAE IN THE FIELD[a]

| Compound | Mean amount (dry weight) | SD | Coefficient of variation (%) |
|---|---|---|---|
| Farinosin | 49.24 mg/g | 14.52 | 29.5 |
| Encecalin | 193.41 mg/g | 81.91 | 42.4 |
| Euparin | 10.41 mg/g | 3.78 | 36.3 |
| Nitrogen | 4.83% | 0.83 | 16.9 |

[a] See text for details.

a mixture of first, second, and third instars showed no preference. The third group, mainly third instars, clearly preferred the high-concentration diet. Such a change in preference could reflect a changing ability to tolerate increasing natural product concentrations as larvae age (Gould and Hodgson, 1980).

To establish the concentrations of the three natural products in the *E. farinosa* leaves preferred by larvae in the field, leaves were collected that had active larvae feeding on them at the time of collection. This avoided any complications with seasonal changes in concentrations in leaves picked after herbivory had ended. These measurements demonstrated no clear-cut expression of preference by the larval population (Table 5). The mean values of the natural product concentrations in the field larvae selected leaves were in the middle of the total range of the concentrations expressed. The dispersion of values, measured by the coefficient of variation, shows a large spread in the concentrations of eaten leaves for these three compounds. Nitrogen measurements of these leaves showed that the larvae selected leaf material with values consistently at the high end of *E. farinosa*'s range (Table 5). Wisdom and Rodriguez (1982, 1983) established the seasonal range of natural product concentrations. In this investigation, larval leaf choices were spread over the range of concentrations, lacking only the highest and lowest concentrations.

## DISCUSSION

Differential herbivory has been linked to differential plant chemical content in several recent studies. Farentinos et al. (1981) found that tassel-eared squirrels actively selected ponderosa pine tree twigs with lower monoterpene content overall and those specifically lower in $\beta$-pinene content. Dolinger et al. (1973) related lycaenid butterfly patterns on lupines to differential chemical content and morphology. Whitham (1981, 1983) has described the role of chemical variation within an individual tree, preventing the evolution of complete detoxification in the insect herbivores. Finally, Zucker (1982) has proposed that total content and gradients of concentrations of phenols relate to reproduction of galling aphids on *Populus*.

Investigation of the natural products (sesquiterpene lactones and chromenes) of *Encelia farinosa* by Wisdom and Rodriguez (1982, 1983) has shown extensive quantitative variation with respect to leaf age, season, individual, population, and geographic location. While both chemical types are reputed to be biologically active against insects (Burnett et al., 1974, 1977a,b, 1978a,b; Jones et al., 1979; Bowers et al., 1976; Bowers, 1976, 1981), only chromenes proved to have significant activity against *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae), the corn earworm (Wisdom et al., 1983). The extracts of *E. farinosa* leaves used in the feeding experiments contained both chemical classes, of which approximately 85% was encecalin, the principal chromene of this plant.

Kraft and Denno (1982) found *Trirhabda baccharidis* insensitive to any seasonal changes in its host plant's natural products. In comparison, O'Brien (1980) found larvae of *T. sericotrachyla* to be sensitive to morphological differences (with no chemical data available) in sympatric populations of its host plant, *Artemisia californica* (Asteraceae). O'Brien measured significant decreases in fecundity when *T. sericotrachyla* were switched to foliage of morphs found at a low percentage of their host population. This is most likely an example of host race evolution.

The field investigation of *T. geminata* larvae indicated differential herbivory. Considering plants individually (larvae not leaving the plant they were oviposited on), different branches showed large differences in larval activity with regards to both leaf damage and larval presence (leaf damage varying from 10% to 95% of leaves per branch). Examination of larval movements (marking branches and with time-lapse photography) indicated that during a 48-hr period, these larvae moved extensively within a plant. Thus, larval aggregations were the result of larval movement and may reflect the active choice of leaf material by larvae and not the sole result of the ovipositing adult female. Larval choices of natural product concentrations in the field showed no relationship between larval distributions and chemical distributions. Larvae restrict their feeding efforts to the top 5–10 leaves per branch, the area which has the highest nitrogen content (Wisdom and Rodriguez, 1983). Nitrogen content could be a major factor in feeding choices of these larvae. Lumping the results of the three agar diet choice experiments failed to reveal any clear-cut preference for high- or low-extract concentrations (nitrogen content being equal in each diet type). On the whole, about half the larvae chose the low-concentration diet and the other half chose the high diet. However, considering the experiments individually as the season progressed, larvae appeared to switch from an initial preference for low concentrations to a preference for high concentrations. This progression corresponds loosely with the seasonal changes in the natural products of *Encelia farinosa* (Wisdom and Rodriguez, 1982, 1983). The simplest explanation is that the larvae are choosing the most nitrogen-rich foliage (similar to the findings in Lincoln et al., 1982), and tolerating the presence of the natural products and the resultant effects on their growth and survivorship.

In contrast to the choice experiments, the high/low diet feeding trials gave clear-cut results. First-instar larvae fed on unlimited low-concentration diet material grew faster and survived better than those larvae on the high diet. This is significant as *Trirhabda geminata* is a specialist herbivore feeding solely on *Encelia farinosa*. No such response was found for the specialist herbivore, *T. baccharidis* feeding on *Baccharis halimifolia* (Kraft and Denno, 1982). This slowed growth, in conjunction with increasing parasitism levels and general predation by a pentatomid, *Perillus splendidus* Uhler (Hemiptera: Pentatomidae), could have a large impact on the *Trirhabda geminata* larval population.

Whitham has summarized the advantages of quantitative variation (differential plant quality) to a target plant (Whitham, 1981). These advantages are: (1) making the plant less apparent to its herbivore, increasing incorrect ovipositional and feeding choices by insect herbivores; (2) increasing competition between insects for superior host resources; and (3) causing the clumping of herbivores, making them more vulnerable to predators and parasites. In this investigation, I have demonstrated that larvae are unable to clearly distinguish between natural product concentrations. Laboratory choice expression tests and measurement of field-selected leaves support this conclusion. Consequently, the role of the natural product variation in *E. farinosa* appears to involve a decrease in some aspects of the apparency of the nutritional quality of leaves to the beetle larvae. Secondly, the action of high concentrations of these natural products is to slow the growth of larvae. Concurrently, the numbers of successfully parasitized larvae increase as the season progresses. Thus, the mechanism of action of the *E. farinosa* natural products appears to be a slowing of beetle growth while vulnerability to parasitism is increasing, enhancing the effectiveness of the beetles' enemies. The summer deciduous *Encelia farinosa* seems to present a time "window" of availability to the beetle larvae since no beetles may feed until the flush of new growth appears in mid-January. Late emerging beetles are at risk to the high parasitism rates in this system. The growth-retarding activity of the plant's natural products will only increase this vulnerability. This may also be an example of the general scenario developed by Price et al. (1980), in which they proposed that plant defense systems could influence the effectiveness of the predators and parasitoids of herbivorous insects. This interaction between *Encelia farinosa* and the larvae of *Trirhabda geminata* appears to be an example of a plant defense system of quantitative variation which retards larval growth and adaptation, enhancing the predation and parasitism pressure of the beetles' natural enemies (as described by Sturgeon, 1981).

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# EFFECTS OF FERULIC AND *p*-COUMARIC ACIDS IN NUTRIENT CULTURE OF CUCUMBER LEAF EXPANSION AS INFLUENCED BY pH[1]

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**Abstract**—Cucumber seedlings were grown in 5 mM MES [2-(*N*-morpholino)ethanesulfonic acid] -buffered nutrient solutions adjusted to a pH of 5.5, 6.25, or 7.0. Nutrient solutions were changed on alternate days. Seedlings were treated for a two-day period with various concentrations (0–1 mM) of ferulic acid, *p*-coumaric acid, or mixtures of these phenolic acids when 16 days old. Leaf growth, dry weight, and water utilization of the seedlings; pH of the solutions; and disappearance of the phenolic acids from nutrient solutions were monitored. Leaf area expansion of cucumber seedlings was inhibited by both ferulic and *p*-coumaric acid, and the magnitude of these inhibitions was influenced by concentration and pH. Inhibition of leaf area expansion was greater at pH 5.5 and nominal at pH 7.0. Ferulic acid was more inhibitory than *p*-coumaric acid. The effect of pH on growth was best described by data for mean relative rates of leaf expansion. For example, the mean relative rates of leaf expansion by both acids at 0.5 mM for the 16- to 18-day growth period (treatment period) were reduced by 45, 31, and 8% for the pH 5.5, 6.25, and 7.0 treatments, respectively. The dry weight of seedlings at harvest (day 22) was significantly reduced for seedlings grown in the pH 5.5 and 6.25 treatments, but not for the pH 7.0 treatment. There was, however, one exception; the dry weight of seedlings treated with *p*-coumaric acid solutions adjusted to a pH of 5.5 was not significantly reduced. Water utilization by the seedlings was reduced by both ferulic and *p*-coumaric acid. Again, the impact of ferulic acid was greater than *p*-coumaric acid. The effect of ferulic acid on water utlization decreased with increasing pH of the nutrient solution. The pH effects were not so consistent for *p*-coumaric acid. The effects of equimolar

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mixtures of the two phenolic acids were additive for all variables measured. There was a linear correlation between mean relative rates of leaf expansion and water utilization.

**Key Words**—Ferulic acid, *p*-coumaric acid, allelopathy, pH, leaf area expansion, phytotoxins, water utilization, cucumber seedlings, *Cucumus sativus*.

## INTRODUCTION

In previous publications we described how ferulic acid, a potential allelopathic agent, and its microbial metabolic products inhibited germination, radicle growth, leaf area expansion, and dry matter production of cucumber (Blum and Dalton, 1985; Blum et al., 1984, 1985). The effects of varying concentrations of these phenolic acids were tested on seedlings in nutrient solutions adjusted to a pH of 5.8. Harper and Balke (1981) noted that the inhibition of $K^+$ absorption in oat root tissue by ferulic acid, a cinnamic acid derivative, and salicylic acid, a benzoic acid derivative, was affected not only by concentration but also pH. The inhibitory effects were reduced with increasing pH. They also noted that absorption of salicylic acid by root segments increased as the pH of treatment solutions decreased to pH 4.5 where root damage occurred. Their observations suggested that the inhibitory effects on growth of cucumber seedlings caused by ferulic acid and some of its microbial metabolic products might also be influenced by pH. The objective of the present research was to explore the effect of solution pH on the inhibition of the growth of cucumber seedlings caused by ferulic and *p*-coumaric acid. Both of these phenolic acids have been implicated as potential allelopathic agents (Rice, 1984) and have been isolated from soils (Whitehead et al., 1983).

## METHODS AND MATERIALS

*General Aspects.* Cucumber seeds (*Cucumis sativus* cv "Early Green Cluster"; Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated in the dark at 28–30°C in trays containing sterile vermiculite and full-strength Hoagland's solution (Hoagland and Arnon, 1950) buffered with MES [2-(*N*-morpholino)ethanesulfonic acid]. After 48 hr, seedlings were transferred to 120-ml glass snap-cap bottles containing full-strength Hoagland's solution (Blum and Dalton, 1985). Nutrient solutions in the bottles were changed on alternate days. Seedlings were grown under three light banks that provided 150 $\mu$ Einsteins/$m^2$/sec for 12 hr per day. Temperatures under the light banks ranged from 21 to 30°C for all experiments. Seedlings were treated with phenolic acids when 14 or 16 days old. Phenolic acids were obtained from Sigma Chemical Company, St. Louis, Missouri. Length and width of each leaf were measured each time nutrient solutions were changed. Dry weights of seedlings (50°C, 48 hr) were determined at harvest.

*Preliminary Studies.* MES buffer (0, 5, or 10 mM) was added to the nu-

trient solutions to determine a level of MES buffer that would stabilize the pH values of the nutrient solutions without affecting seedling growth. The pH values of the initial solutions were adjusted to 5.5 and 7.0. pH values were determined just before each nutrient solution change. After 14 days, seedlings were treated with 0 or 0.5 mM ferulic or *p*-coumaric acid dissolved in nutrient solution. Seedlings were kept in these phenolic acid solutions for two days. Seedlings were harvested when 20 days old ($N = 3$, total number of plants = 90).

In a subsequent experiment cucumber seedlings were grown in nutrient solutions buffered with 5 mM MES which were initially adjusted to a pH of 5.5, 6.25 or 7.0. Seedlings (one plant per treatment) were treated with 0.5 mM ferulic or *p*-coumaric acid when 16 days old. Levels of the phenolic acids were monitored at 1, 3, 6, 24, and 48 hr by injecting samples of these solutions into a Waters fully automated high-performance liquid chromatograph (Blum et al., 1985). The height of the solution level in each bottle was mesured at 6, 24, and 48 hr. Bottles containing ferulic acid (0.5 mM) dissolved in nutrient solutions (pH 5.5, 6.25, or 7.0), but without seedlings, were also monitored. Bottles were sterilized and the nutrient solutions containing the phenolic acids were filter-sterilized. pH values of the solutions were monitored at the same time intervals for an additional set of plants treated with ferulic acid.

*pH Study.* Seedlings were grown in nutrient solutions buffered with 5 mM MES which were initially adjusted to a pH of 5.5, 6.25, or 7.0. When 16 days old seedlings were treated with ferulic acid, *p*-coumaric acid, and mixtures of ferulic and *p*-coumaric acids dissolved in nutrient solution. Seedlings were removed from these solutions after two days ($N = 3$, total number of plants = 81). Concentrations used were 0, 0.25, 0.5, and 1.0 mM for the single phenolic acid treatments and 0.25 and 0.5 mM for the equimolar mixtures. In addition to the leaf and solution pH measurements, the height of the solution in each bottle was also measured just before each solution change. Plants were harvested when 22 days old.

*Data Analysis.* Unless otherwise stated, all treatments ($N = 3$) for each experiment were randomly distributed under each of the three light banks. Data were analyzed using the Statistical Analysis System (SAS) programs for analysis of variance and linear and multiple regressions (SAS Institute Inc., 1982). Least significant differences ($LSD_{0.05}$), were presented, are provided only as a measure of experimental precision and inferences are based on the appropriate analysis of variance or regression analysis.

Leaf areas were determined from leaf length and width measurements and the following equation: leaf area $= -1.457 + 0.00769(L \times W)$, $\alpha = 0.0001$, $r^2 = 0.98$, $N = 121$ where leaf area is in $cm^2$ and length ($L$) and width ($W$) values are in mm (Blum and Dalton, 1985). The mean absolute rates of leaf expansion were determined by the equation: mean absolute rate = leaf area at time$_{x+1}$ − leaf area at time$_x$. Since all readings were taken at two-day intervals, values given are based on this interval. Mean relative rates of leaf expansion were determined by the equation: mean relative rates = ln(leaf area

at $time_{x+1}$ + 1) − ln(leaf area at $time_x$ + 1). These values were also based on two-day intervals. Volumes of the solutions in the snap-cap bottles were determined from solution height and the following equation: ml of solution = 13.78 + 1.5(height in mm), $\alpha = 0.0001$, $r^2 = 0.99$, $N = 36$. Water utilization of a seedling was determined by dividing the milliliters of water lost by transpiration over a given two-day period by the leaf area of that seedling on day 2.

## RESULTS

*Preliminary Study.* The pH values of the nutrient solutions were significantly modified by cucumber seedlings over 48-hr periods (Figure 1). Previously, we had used MES buffer to stabilize the pH of the phenolic acid solutions without obvious effects on radicle growth of cucumbers (Blum et al., 1984). The addition of MES buffer [$pK_a$ (20°C) = 6.15] to nutrient solutions partially stabilized the pH values of the nutrient solutions over the 48-hr periods. There was, however, a significant buffer by pH treatment interaction. The buffer stabilized the 5.5 pH values initially, but as the plants became larger, the ability of the buffer to stabilize the pH of the nutrient solutions declined. The MES buffer maintained the pH 7.0 solutions above 6.6 for all 48-hr periods (Figure 1).

To determine how MES buffer, pH of the nutrient solution, and phenolic acids in the solutions might interact and affect leaf expansion, 14-day-old cucumber seedlings that had grown in 0, 5, or 10 mM MES buffered nutrient solutions adjusted to pH 5.5 or 7.0 were treated with 0 or 0.5 mM ferulic or *p*-coumaric acid. Leaf area and absolute rates of leaf expansion over the 14- to 16-day growth period (treatment period) were modified by pH, MES buffer, and the phenolic acids. Leaf area and mean absolute rates of leaf expansion were approximately 22% larger when seedlings were grown at pH 7.0 compared to pH 5.5 and 3 and 22% (significant at 0.05) smaller in the 5 and 10 mM MES buffer treatments, respectively, compared to seedlings grown in the nonbuffered nutrient solutions. Leaf area was reduced 15 and 18%, and mean absolute rates of leaf expansion were reduced 39 and 33% by ferulic and *p*-coumaric acid treatments, respectively. The mean leaf area and mean absolute rates of leaf expansion for control plants were 74 $cm^2$ and 33 $cm^2$/2 days, respectively ($LSD_{0.05}$ = 3).

Based on the foregoing results, we used 5 mM MES buffer to stabilize nutrient solution pH in further experiments. The improvement in the stability of pH by 10 mM MES buffer was counterbalanced by the significant reductions in leaf area and absolute rates of leaf expansion of the seedlings.

Root absorption of either ferulic acid or *p*-coumaric acid (0.5 mM) from nutrient solutions over the first 3- or 6-hr appeared to be influenced by pH (Figure 2). This corresponded to what Harper and Balke (1981) noted for the absorption of [$^{14}$C]salicylic acid by root segments. In our case, however, the presence of microbes in the nutrient solutions containing phenolic acids (nonlabeled)

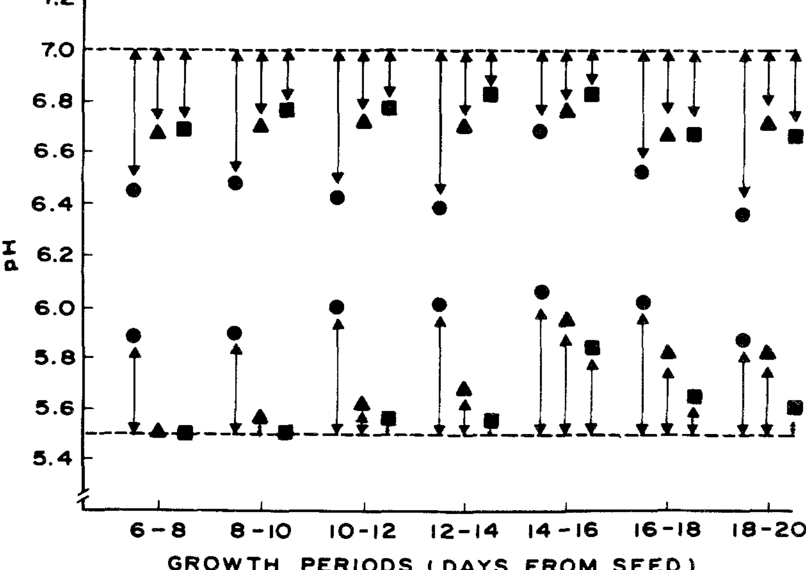


FIG. 1. Changes in pH during 48 hr in nutrient solutions containing 0, 5, or 10 mM MES buffer in which cucumber seedlings of various ages have been grown. Initial pH values of the solutions are indicated by the dashed lines ($N = 15$).

make it difficult, if not impossible, to state that the disappearance observed beyond 3–6 hr was largely due to absorption by cucumber roots.

The stability of ferulic acid (only one tested) in nutrient solutions placed under the light banks in bottles without plants and microbes appeared also to be pH dependent. After 6 hr, 9, 7, and 3% of the ferulic acid disappeared from the bottles with the pH 5.5, 6.25, and 7.0 treatments, respectively. At 48 hr these losses amounted to 22, 14, and 10%. Since light reaching the solutions in the bottles without plants was considerably greater than the solutions in the bottles with plants, these estimates of degradation were probably an overestimation.

*pH Study.* The 48-hr pH values of the nutrient solutions for the last three growth periods ranged from 5.7 to 6.3, 6.3 to 6.9, and 6.6 to 7.1 for the initial pH treatments of 5.5, 6.25, and 7.0 pH, respectively. For example, mean 48-hr

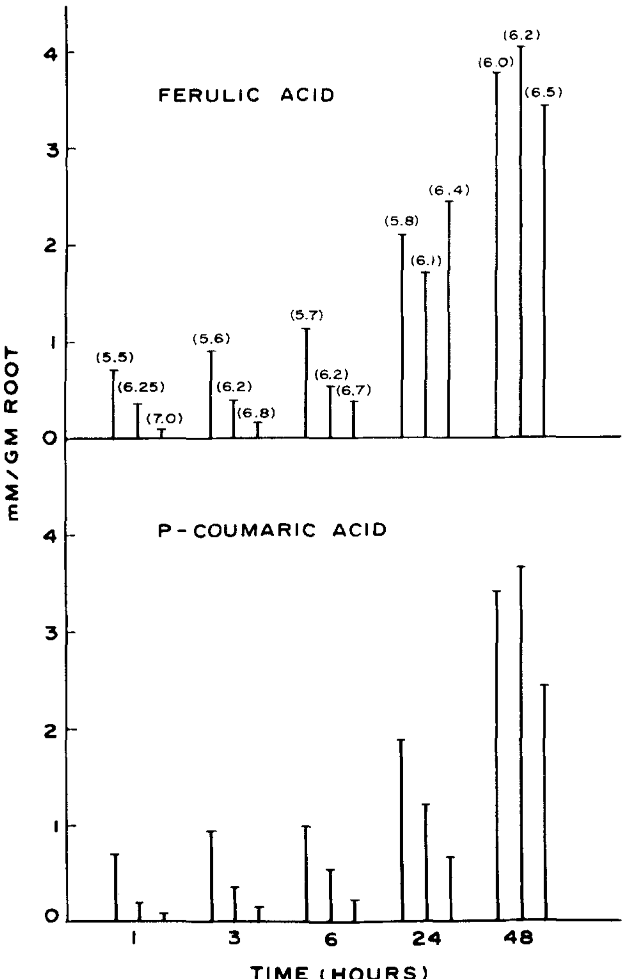


FIG. 2. Disappearance of ferulic and *p*-coumaric acids from nutrient solutions containing 16- to 18-day-old cucumber seedlings. pH values (48 hr) for the nutrient solutions are given in parentheses. The pH values were similar for both phenolic acids. Initial pH values were 5.5, 6.25, and 7.0 Data are given in this order. Root dry weights and standard errors of plants growing in ferulic and *p*-coumaric acid were 0.133 ± 0.012 and 0.144 ± 0.009, respectively. Values are presented on a root dry weight basis since the disappearance over the first 6 hours was most likely due to root uptake. Microbial metabolism appeared to become more important beyond 6 hr.

pH values for the 16- to 18-day growth period (treatment period) were 6.1, 6.7, and 6.9 for the respective 5.5, 6.25, and 7.0 pH treatments ($LSD_{0.05}$ = 0.14). These values are similar to those obtained with the 5 mM MES buffered nutrient solutions of the preliminary experiment. Figure 2 indicates that shifts in solution pH occurred slowly over time and that different pH values, although small at 48 hr (time of solution change), were still present for the three initial pH treatments.

Leaf area and dry weight of cucumber seedlings to an age of 24 days were linearly correlated (Blum et al., 1985). Since leaf area can be easily obtained without destructive sampling and is a rapid reliable indicator of plant response to phenolic acids, we chose to utilize this seedling parameter for study. From information presented in Figure 3 and Tables 1, 2, and 3, it appeared that leaf expansion of cucumber seedlings: (1) was inhibited in a concentration-dependent manner by both ferulic acid and *p*-coumaric acid, (2) was inhibited more by ferulic acid than by *p*-coumaric acid, (3) was inhibited the most at pH 5.5 and least, if at all, at pH 7.0, and (4) was rapidly reinitiated once the phenolic acids were removed from the root environment. However, as to the last findings, it should be noted that recovery of mean relative rates of leaf expansion has also been observed in the continued presence of phenolic acids (Blum and Dalton, 1985; Blum et al., 1985). The effects of pH on growth (Figure 3) can most clearly be seen in the data for mean relative rates of leaf expansion ($cm^2/cm^2/2$ days). For example, the mean relative rates of leaf expansion by seedlings exposed to either phenolic acid at 0.5 mM during the 16- to 18-day growth period were reduced 45, 31, and 8% for the pH 5.5, 6.25, and 7.0 treatments, respectively. The reduction in leaf area and seedling dry weight at harvest (day 22) were significant for seedlings grown in the pH 5.5 and 6.25 treatments, but not for the pH 7.0 treatment (Tables 1, 2, and 3). There was however, one exception to the above statement; the dry weights of seedlings treated with *p*-coumaric acid at pH 5.5 was not significantly reduced.

The effects of the equimolar mixtures of ferulic and *p*-coumaric acid were additive for all plant variables measured. The effects of the mixtures were essentially equal to those expected based on modeling procedures (Blum et al., 1984, 1985). Table 4 presents percent reductions for mean relative rates of leaf expansion for ferulic acid, *p*-coumaric acid, and mixtures of these acids. The difference between the mean sum values (expected) and the mixture values was not significantly different.

Water utilization ($ml/cm^2$ leaf area/2 days) was reduced by both ferulic and *p*-coumaric acids (Figure 4, Tables 5 and 6). The impact of ferulic acid was greater than that of *p*-coumaric acid. The effects of ferulic acid on water utilization decreased with increasing pH of the nutrient solution. The pH effects were not as consistent for *p*-coumaric acid. Leaves of seedlings treated with 0.5 mM or higher concentrations of phenolic acids grown in nutrient solutions at a

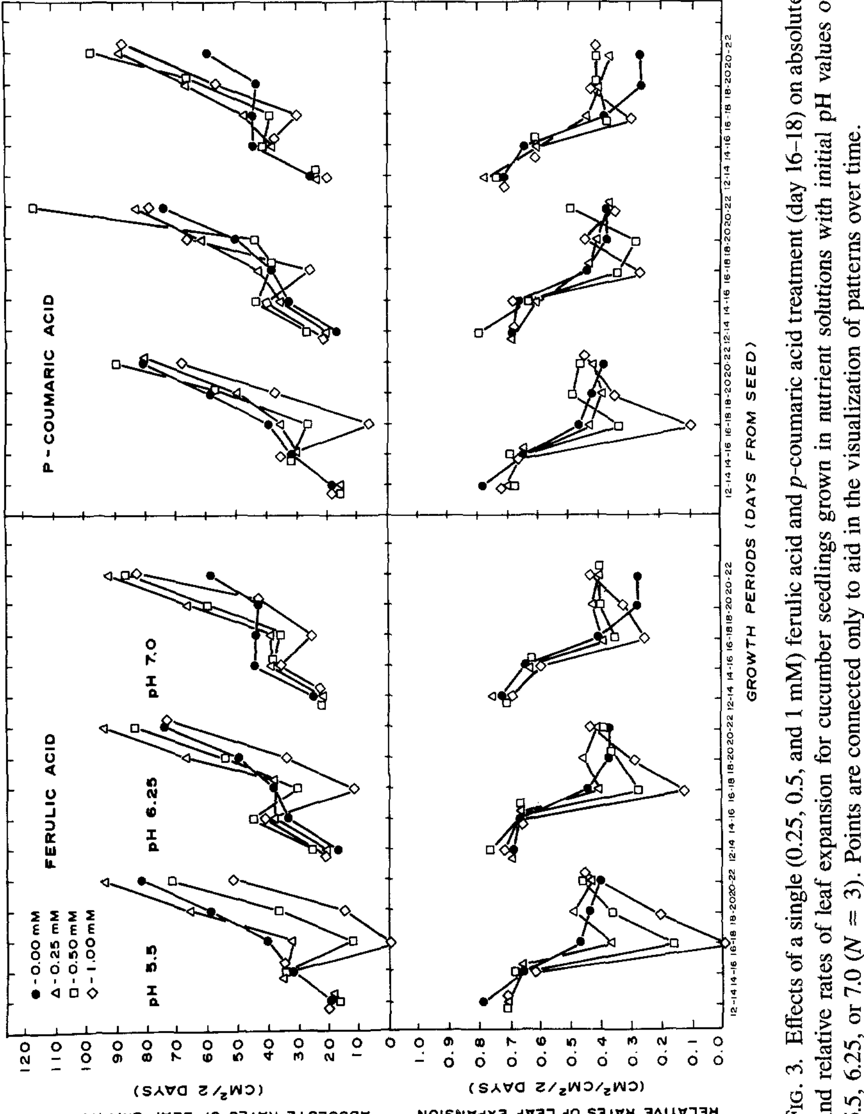


FIG. 3. Effects of a single (0.25, 0.5, and 1 mM) ferulic acid and *p*-coumaric acid treatment (day 16–18) on absolute and relative rates of leaf expansion for cucumber seedlings grown in nutrient solutions with initial pH values of 5.5, 6.25, or 7.0 ($N = 3$). Points are connected only to aid in the visualization of patterns over time.

TABLE 1. SUMMARY OF MEAN SQUARE VALUES FROM ANALYSIS OF VARIANCE FOR LEAF AREA, PLANT DRY WEIGHT, AND MEAN ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION.[a]

| | | Leaf area | | | Dry weight | Mean absolute rates of leaf expansion | | Mean relative rates of leaf expansion | |
|---|---|---|---|---|---|---|---|---|---|
| | | Age (days) | | | | Growth periods (days) | | | |
| | *df* | 18 | 20 | 22 | 22 | 16–18 | 18–20 | 16–18 | 18–20 |
| Phenol[b] | 1 | 524*[c] | 2031* | 4444 | 0.0383 | 670* | 491 | 0.0728* | 0.0197 |
| Conc | 3 | 1480* | 4691* | 9510* | 0.0783* | 2233* | 1229* | 0.2575* | 0.0255* |
| pH | 2 | 5978* | 9425* | 12671* | 0.1788* | 1238* | 390 | 0.0366* | 0.0057 |
| Phenol × Conc | 3 | 102 | 1141 | 3102 | 0.0189 | 86* | 699* | 0.0093* | 0.0269* |
| Phenol × pH | 2 | 10 | 2 | 19 | 0.0026 | 19 | 13 | 0.0071 | 0.0026 |
| Conc × pH | 6 | 614* | 1183* | 3119* | 0.0256* | 167* | 537* | 0.0292* | 0.0244* |
| Phenol × Conc × pH | 6 | 18 | 301 | 346 | 0.0040 | 23 | 196 | 0.0032 | 0.0068 |
| Error | 48 | 124 | 413 | 1149 | 0.0097 | 30 | 173 | 0.0027 | 0.0057 |

[a] Seedlings were grown in nutrient solution with initial pH values of 5.5, 6.25, or 7.0. They were treated with ferulic acid or *p*-coumaric acid when 16–18 days old ($N = 3$).
[b] Phenol: ferulic and *p*-coumaric acids; Conc = 0, 0.25, 0.5, and 1.0 mM; pH: initial pH of 5.5, 6.25, or 7.0.
[c] Significance level*: $\leqslant$ 0.5 level of probability.

TABLE 2. PARTIAL REGRESSION COEFFICIENT AND $R^2$ VALUES FOR LEAF AREA, PLANT WEIGHT, AND MEAN ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS GIVEN A SINGLE FERULIC ACID TREATMENT WHEN 16–18 DAYS OLD (CONCENTRATIONS RANGED FROM 0 TO 1 mM).

| Age or growth period (days) | pH | Intercept | Linear | Quadratic | $R^2$ |
|---|---|---|---|---|---|
| Leaf area ($cm^2$) | | | | | |
| 22 | 5.5 | 263.79 | −119.37 | | 0.65 |
| | 6.25 | 235.53 | 178.75 | −207.75 | 0.47 |
| | 7.0 | — | — | — | NS[a] |
| Plant dry weight (g) | | | | | |
| 22 | 5.5 | 0.86 | −0.35 | | 0.65 |
| | 6.25 | 0.77 | 0.51 | −0.54 | 0.29 |
| | 7.0 | — | — | — | NS |
| Mean absolute rates of leaf expansion ($cm^2$/2 days) | | | | | |
| 16–18 | 5.5 | 40.04 | −43.75 | | 0.90 |
| | 6.25 | 41.88 | −27.24 | | 0.69 |
| | 7.0 | 44.97 | −19.14 | | 0.72 |
| 18–20 | 5.5 | 66.19 | −48.44 | | 0.73 |
| | 6.25 | — | — | — | NS |
| | 7.0 | 45.82 | 78.04 | −80.37 | 0.33 |
| Mean relative rates of leaf expansion ($cm^2/cm^2$/2 days) | | | | | |
| 16–18 | 5.5 | 0.47 | −0.51 | | 0.94 |
| | 6.25 | 0.45 | −0.32 | | 0.78 |
| | 7.0 | 0.42 | −0.14 | | 0.58 |
| 18–20 | 5.5 | 0.49 | −0.26 | | 0.59 |
| | 6.25 | — | — | — | NS |
| | 7.0 | 0.28 | 0.57 | −0.52 | 0.43 |

[a] NS = not significant at $\leqslant$ 0.05 level of probability.

pH of 5.5 wilted within 1–2 hr. Recovery of leaves occurred within 24–48 hr. The pattern of water utilization for both phenolic acids (Figure 4) appeared to be similar to those of mean relative rates of leaf expansion (Figure 3). The linear correlation coefficient for mean relative rates of leaf expansion and water utilization (all treatments included; $r \doteq 0.91$ for ferulic acid; $r = 0.82$ for *p*-coumaric acid) were significant ($P < 0.0001$). The linear correlation coefficients after treatment effects were removed were no longer significant for ferulic

TABLE 3. PARTIAL REGRESSION COEFFICIENT AND $R^2$ VALUES FOR LEAF AREA, PLANT WEIGHT, AND MEAN ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS GIVEN A SINGLE *p*-COUMARIC ACID TREATMENT WHEN 16-18 DAYS OLD (CONCENTRATIONS RANGED FROM 0 TO 1 mM).

| Age or growth period (days) | pH | Intercept | Linear | Quadratic | Cubic | $R^2$ |
|---|---|---|---|---|---|---|
| Leaf area ($cm^2$) | | | | | | |
| 22 | 5.5 | 252.07 | −55.95 | | | 0.35 |
| | 6.25 | 229.07 | 225.61 | −197.80 | | 0.47 |
| | 7.0 | — | — | — | | NS[a] |
| Plant dry weight (g) | | | | | | |
| 22 | 5.5 | — | — | — | | NS |
| | 6.25 | 0.76 | 0.78 | −0.67 | | 0.54 |
| | 7.0 | — | — | — | | NS |
| Mean absolute rates of leaf expansion ($cm^2$/2 days) | | | | | | |
| 16–18 | 5.5 | 42.75 | −33.73 | | | 0.90 |
| | 6.25 | 38.79 | 21.48 | −33.75 | | 0.70 |
| | 7.0 | 47.88 | −16.50 | | | 0.64 |
| 18–20 | 5.5 | — | — | — | | NS |
| | 6.25 | — | — | — | | NS |
| | 7.0 | 45.50 | 82.92 | −70.03 | | 0.36 |
| Mean relative rates of leaf expansion ($cm^2/cm^2$/2 days) | | | | | | |
| 16–18 | 5.5 | 0.48 | −0.11 | −0.27 | | 0.90 |
| | 6.25 | 0.46 | −0.18 | | | 0.74 |
| | 7.0 | 0.44 | −0.12 | | | 0.47 |
| 18–20 | 5.5 | — | — | — | | NS |
| | 6.25 | 0.38 | 0.80 | −3.32 | 2.60 | 0.51 |
| | 7.0 | — | — | — | | NS |

[a]NS = not significant at $\leqslant$ 0.05 level of probability.

acid, and considerably reduced in significance for *p*-coumaric acid ($r = 0.49$, $P < 0.013$). The linear models (all treatments included) for mean relative rates of leaf expansion ($R$) and water utilization ($W$) were as follows for the 16- to 18-day growth period:

Ferulic acid

$$R = -0.104 + 1.2(W),\ \alpha = 0.0001,\ N = 36,\ r^2 = 0.87$$

TABLE 4. PERCENT REDUCTION FOR MEAN RELATIVE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS FOR GROWTH PERIOD 16–18 DAYS TREATED ONCE WITH FERULIC OR *p*-COUMARIC ACID AND MIXTURES OF THESE PHENOLIC ACIDS.[a]

| pH | mM | Ferulic acid | *p*-Coumaric acid | Sum | Mixture | Sum − mixture |
|---|---|---|---|---|---|---|
| 5.5 | 0.25 | 23 | 6 | 29 | 28 | 1 |
| | 0.5 | 65 | 26 | 91 | 87 | 4 |
| | 1.0 | 100 | 79 | | | |
| 6.25 | 0.25 | 9 | 0 | 9 | 18 | −9 |
| | 0.5 | 39 | 20 | 59 | 57 | 2 |
| | 1.0 | 70 | 36 | | | |
| 7.0 | 0.25 | 2 | 0 | 2 | 10 | −8 |
| | 0.5 | 8 | 8 | 16 | 22 | −6 |
| | 1.0 | 35 | 22 | | | |

[a] Seedlings were treated once when 16 days old. Nutrient solutions were changed every other day. Mean relative rates of leaf expansion of control seedlings were: pH 5.5 = 0.47 $cm^2/cm^2/2$ days, pH 6.25 = 0.44, pH 7.0 = 0.40.

*p*-Coumaric acid

$$R = -0.175 + 1.35(W),\ \alpha = 0.0001,\ N = 36,\ r^2 = 0.73$$

where $R$ is in $cm^2/cm^2/2$ days and $W$ is in $ml/cm^2/2$ days. The effects of the equimolar mixtures of ferulic and *p*-coumaric acids on water utilization were additive.

## DISCUSSION

We have demonstrated that the pH of the root environment of cucumber seedlings alters the inhibitory activity of both ferulic and *p*-coumaric acid. The inhibitory activity of these two phenolic acids on growth of cucumber seedlings declined dramatically with increasing pH. A pH range of 6.0–6.5 is recommended for best growth of cucumber plants (Hughes et al., 1983). The pH treatments for this study ranged from 5.5 to 7.0. It is likely that the main effects of solution pH are on the modification of the solubility and ionization of these phenolic acids. The pKa of ferulic acid is approximately 4.8. Ferulic acid solubility increases and more ferulic acid molecules become negatively charged as pH of the solution increases. Harper and Balke (1981) concluded from their studies with salicylic acid (pKa = 3) and oat root tissue that membranes were more permeable to the undissociated form of salicylic acid. This appeared to be the case for both ferulic and *p*-coumaric acid in the present study, since the

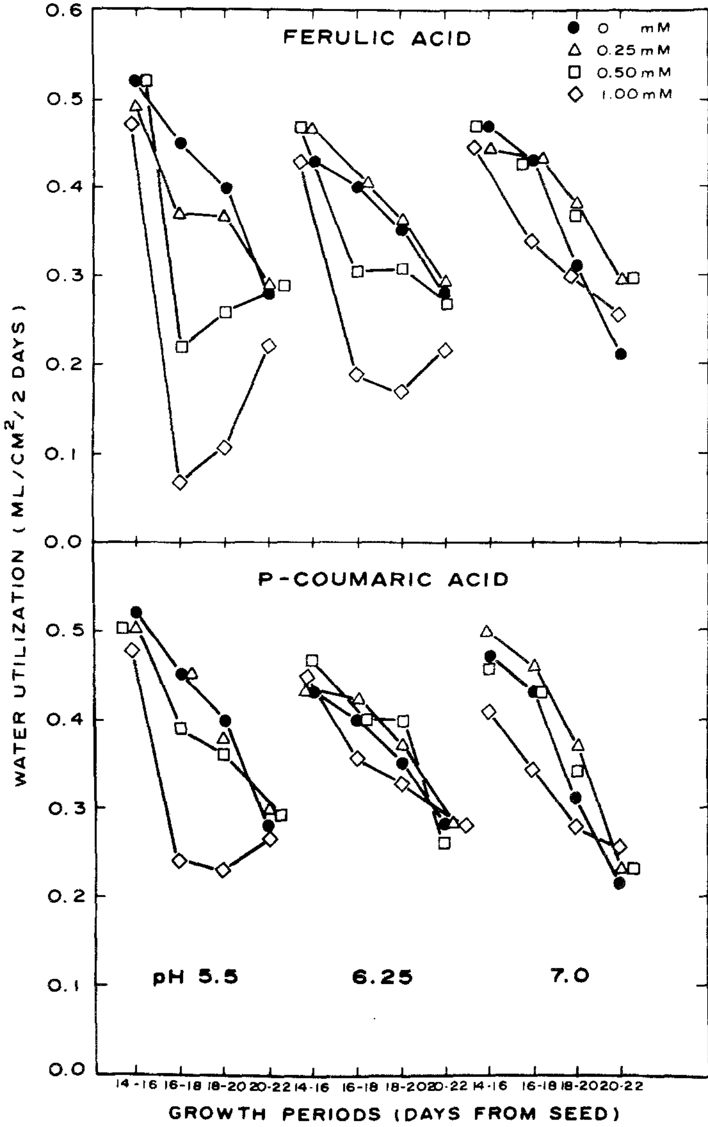


FIG. 4. Effects of a single (0.25, 0.5, and 1 mM) ferulic acid and *p*-coumaric acid treatment (day 16–18) on water utilization of cucumber seedlings grown in nutrient solutions with initial pH values of 5.5, 6.25, or 7.0 ($N = 3$). Points are connected only to aid in the visualization of patterns over time.

TABLE 5. SUMMARY OF MEAN SQUARE VALUES FROM ANALYSIS OF VARIANCE OF WATER UTILIZATION.[a]

| | *df* | Growth periods (days) | | |
|---|---|---|---|---|
| | | 16–18 | 18–20 | 20–22 |
| Phenol | 1 | 0.0718* | 0.0259* | 0.0013 |
| Conc | 3 | 0.0114* | 0.0637* | 0.0062* |
| pH | 2 | 0.0386* | 0.0022 | 0.0022 |
| Phenol × Conc | 3 | 0.0113* | 0.0077* | 0.0013 |
| Phenol × pH | 2 | 0.0142* | 0.0104* | 0.0010 |
| Conc × pH | 6 | 0.0136* | 0.0122* | 0.0028* |
| Phenol × Conc × pH | 6 | 0.0039* | 0.0033 | 0.0003 |
| Error | 48 | 0.0013 | 0.0025 | 0.0011 |

[a] See Table 1.

TABLE 6. PARTIAL REGRESSION COEFFICIENT AND $R^2$ VALUES OF WATER UTILIZATION ($ml/cm^2$/2 DAYS) FOR CUCUMBER SEEDLINGS GIVEN A SINGLE PHENOLIC ACID TREATMENT WHEN 16–18 DAYS OLD (CONCENTRATIONS RANGED FROM 0 TO 1 mM).

| Growth Period (Days) | pH | Phenolic acid[a] | Intercept | Linear | Quadratic | $R^2$ |
|---|---|---|---|---|---|---|
| 16–18 | 5.5 | | 0.45 | −0.39 | | 0.95 |
| | 6.25 | FER | 0.43 | −0.23 | | 0.88 |
| | 7.0 | | 0.45 | −0.09 | | 0.69 |
| 18–20 | 5.5 | | 0.42 | −0.30 | | 0.90 |
| | 6.25 | FER | 0.38 | −0.19 | | 0.73 |
| | 7.0 | | 0.31 | 0.26 | −0.28 | 0.35 |
| 20–22 | 5.5 | | — | — | — | NS[b] |
| | 6.25 | FER | 0.29 | −0.06 | | 0.36 |
| | 7.0 | | 0.22 | 0.32 | −0.27 | 0.59 |
| 16–18 | 5.5 | | 0.46 | −0.02 | −0.19 | 0.85 |
| | 6.25 | PCO | — | — | — | NS |
| | 7.0 | | 0.44 | 0.11 | −0.21 | 0.59 |
| 18–20 | 5.5 | | 0.41 | −0.17 | | 0.62 |
| | 6.25 | PCO | — | — | — | NS |
| | 7.0 | | — | — | — | NS |
| 20–22 | 5.5 | | — | — | — | NS |
| | 6.25 | PCO | — | — | — | NS |
| | 7.0 | | 0.21 | 0.26 | −0.21 | 0.44 |

[a] FER = ferulic acid, PCO = *p*-coumaric acid.
[b] NS = not significant at ⩽ 0.05 significance level.

greatest disappearance of the phenolic acids from solution culture and the greatest effects on growth were observed at the lowest pH treatment (pH 5.5).

In addition, we have provided data to support our previous observation that ferulic and *p*-coumaric acids reduce water utilization of cucumber seedlings (Blum et al., 1985). The effects of these phenolic acids on water utilization could not be explained by differences in the osmotic potential of the treatment solutions (Blum et al., 1985). The fact that the impact of both phenolic acids on water utilization was greatest at pH 5.5 and that leaves of seedlings wilted only at pH 5.5 when treated with concentrations of 0.5 mM or greater suggested that the phenolic acids, when undissociated, modified water uptake by cucumber roots. It is possible that the primary effect of these phenolic acids on cucumber seedlings is on water uptake and, if so, the observed inhibition of leaf expansion and dry matter production would be secondary. Reduction in leaf expansion would occur as a result of reduced hydrostatic pressure for cell expansion, while reductions in dry matter production would be due to the consequences of stomatal closure and/or disruption of cell metabolism. That phenolic acids bring about stomatal closure has been reported by Patterson (1981), Einhellig and Muth (1980) and Einhellig and Kuan (1971). We noted that treatments of cucumber seedlings with ferulic or *p*-coumaric acid resulted in stomatal closure that lasted for several days (Blum et al., 1985). Patterson (1981) also reported a reduction in the rate of photosynthesis by soybean seedlings when these were treated with ferulic acid. The "water uptake" scenario for cucumber, however, should be viewed with caution, since it is based primarily on the effects of acute levels (0.5 mM and greater) of phenolic acids. The effects of ferulic and *p*-coumaric acid on growth, water utilization, and stomatal aperture (Blum et al., 1985) were abated once the cucumber roots were removed from the inhibitory phenolic acid solutions.

Estimates of individual phenolic acids in soils that are directly available to interact with plant roots are generally presumed to be small (Whitehead et al., 1982). However, soils usually contain an array of phenolic acids including ferulic and *p*-coumaric acid (Whitehead, 1964; Whitehead et al., 1981, 1982). The observed additive inhibitory effects of ferulic and *p*-coumaric acid suggest a potential for additive effects of phenolic compounds in the soil in inhibiting growth of cucumber seedlings. Since the magnitude of inhibition of cucumber growth and water utilization by each phenolic acid was influenced by pH, it was not surprising that the magnitude of inhibition of the mixtures of the phenolic acids was also influenced by pH. The greatest inhibition of the mixtures occurred at pH 5.5.

Finally, based on these observations, we recommend that future seedling studies include some attempt to stabilize the pH of the various test solutions by using an appropriate buffer and changing solutions as frequently as necessary to maintain the desired pH range. This buffer should neither interfere with normal

growth of the bioassay species nor should it interact with the phenolic acid being tested. If this is not possible, then at least the pH of the solutions being tested should be monitored closely over time.

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# RESPONSE SPECIFICITY OF MALE PINK BOLLWORM MOTHS TO DIFFERENT BLENDS AND DOSAGES OF SEX PHEROMONE

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**Abstract**—Male pink bollworm, *Pectinophora gossypiella*, were tested in a sustained flight tunnel to 119 blend–dosage combinations of the sex pheromone components, (*Z*,*Z*)- and (*Z*,*E*)-7,11-hexadecadienyl acetates, which are released by conspecific females in a 61 : 39 ratio. Hierarchical cluster analysis was used to define an area of peak attraction. Within this area males were most sensitive at lower dosages to ratios around the natural blend of components, but exhibited peak response levels at higher dosages to a wide range of blends, with a shift in blend preference to higher *Z*,*Z* isomer ratios at the highest dosage tested (10 mg). Male response specificity appeared to be influenced exclusively by a threshold for initiation of upwind flight, and not arrestment of upwind flight. The results are compared with those of another species, the Oriental fruit moth.

**Key Words**—Sex pheromone, pink bollworm, *Pectinophora gossypiella*, Lepidoptera, Gelechiidae, behavioral thresholds, sustained flight tunnel, odor discrimination, Oriental fruit moth, *Grapholita molesta*, Tortricidae.

## INTRODUCTION

Male pink bollworm moths, *Pectinophora gossypiella* (Saunders) (PBW), respond to a sex pheromone composed of two compounds, (*Z*,*Z*)- and (*Z*,*E*)-7,11-hexadecadienyl acetate (Hummel et al., 1973). Recently, Haynes et al. (1984) showed that conspecific females release this blend in a 61 : 39 ratio of *Z*,*Z* to *Z*,*E* isomers. Field studies have shown that in the early or late part of the season, blends containing 60–67% *Z*,*Z* isomer are most attractive, but that during mid-season a 50 : 50 mix is as good or better than a 60 : 40 blend (Flint et al., 1977,



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1978). Peak male response to ratios varying between 50 and 60% *Z,Z* isomer has also been demonstrated on a worldwide basis (Flint et al., 1979).

In the threshold hypothesis for pheromone perception, Roelofs (1978) suggested that changes in blend preference could be explained by postulating behavioral thresholds for activation of upwind flight and in-flight arrestment that change as a function of blend composition and release rate. These thresholds define an area of peak attraction, with males most sensitive to the natural female produced blend and release rate, but able to respond preferentially to different blends at increased release rates.

The present study was initiated to define the response profiles for the behavioral sequence of PBW males in a sustained-flight tunnel to a series of treatments varying in blend ratio and release rate. The results were used to define behavioral thresholds that control the response profiles. These thresholds, such as for activation of upwind flight and inflight arrestment, could help to explain the seasonal broadening of male response in field studies and also provide a comparison with results from another species, the Oriental fruit moth, *Grapholita molesta* (Busck) (OFM) (Linn and Roelofs, 1983).

## METHODS AND MATERIALS

*Insects.* PBW were reared individually in 2-oz plastic cups on synthetic diet (Adkisson et al., 1960), at 26–27°C, 16:8 hr light–dark, 30–50% relative humidity. Individuals were sexed as pupae and separated from each other. Males were provided with 8% sucrose solution and held under conditions similar to those during rearing until testing during the third or fourth nights as adults.

*Chemicals.* The component isomers were obtained from Albany Int., and were >99% pure, as determined by capillary GLC. Solutions of desired ratios (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100% *Z,Z* isomer) were prepared in Skelly B (predominantly *n*-hexane) and checked by capillary GLC (45-m Carbowax 20 M column). All blends were within ±1% of the desired ratio. A dilution series of each ratio was prepared and dispensed onto rubber septa (Arthur Thomas Co., red, 5 × 9 mm) in 100-μl amounts to achieve dosages of 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 mg per septum. Rubber septa were placed in 4-dram vials and kept at −10°C when not in use.

*Testing Protocol.* Male behavior was observed in the flight tunnel of Miller and Roelofs (1978). Males were placed individually in 4-dram vials and taken to the room housing the tunnel 1 hr prior to the initiation of scotophase, so as to allow them to acclimate to the scotophase light and temperature conditions: 0.3 lux, 26°C. Other flight tunnel conditions were 30% relative humidity, 40 cm/sec wind speed.

Males were observed during the fifth and sixth hours of the 8-hr scotophase (Farkas et al., 1974), after the procedure of Linn and Roelofs (1981). Males

were scored for the following behaviors: activation, wing fanning, and walking; taking flight; orientation in the odor plume; initiation of upwind flight over a 1.5-m distance; and contact with the septum.

Seventy males were observed for each of the 119 treatments. For most of the experimental period two groups of insects were tested per working day (scotophase beginning at 0900 for one group and 1130 hr for the other) and during each 2-hr test period two to four treatments were tested, with no more than 20 males tested per treatment per 2-hr period. The treatments were randomized over the experimental period. ANOVA and regression analysis (Sokal and Rohlf, 1969) were used to show that the data sets for each treatment were not significantly different over the long testing period (see Linn and Roelofs, 1983). Analysis of the data was based on the total number of males tested to each treatment. The table of response values of each treatment was first subjected to hierarchical cluster analysis (as in Linn and Roelofs, 1983), using an average linkage analysis with unweighted means (Sneath and Sokal, 1974), and the similarity coefficient of Gower (1971). The analysis was performed on a Prime 400 computer using a program in the Genstat Statistical Package (Alvey et al., 1977). From the resulting dendrogram, clusters of treatments were analyzed as in Linn and Roelofs (1983).

## RESULTS

The percentage of male PBW completing each of three key behaviors (taking flight, initiation of upwind flight, and source contact) in the flight tunnel to the 119 blend-dosage combinations is shown in Figure 1. The greatest percentage of PBW moths reaching the source (88%) occurred with the 1 mg 65:35 *Z,Z*/*Z,E* blend. Males took flight in high numbers (>80%) to most blends in the dosage range of 0.1–10 mg. Fewer males initiated upwind flight and, with respect to source contacts, the plots also indicate that males reached the source in high percentages (>70%) to a wide range of treatments. In addition, a shift in blend preference occurred as the dosage was increased, from ratios of 60–65% *Z,Z* at 0.1 mg to 40–50% *Z,Z* at 10 mg.

A more detailed analysis was provided by hierarchical cluster analysis. The analysis was based on three of the recorded behaviors (taking flight, upwind flight, and source contact), as these provided all of the essential information for the clustering. From the resulting dendrogram, four clusters were identified, and the pattern of mean response levels over the behavioral sequence for each cluster of treatments are shown in Figure 2. These response patterns show that for each cluster there was no significant difference in the number of source contacts as a function of the number of males initiating upwind flight ($\chi^2$ 2 × 2 test of independence with Yates' correction, $P > 0.05$, Sokal and Rohlf, 1969). For any treatment, male behavior was thus most affected in the early

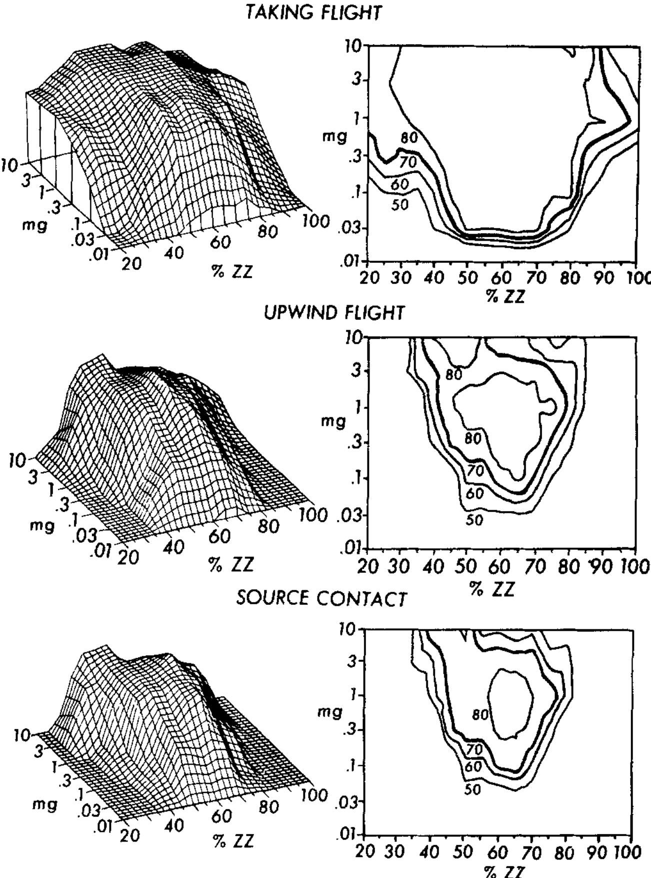


FIG. 1. Response of male PBW for three key behaviors in the flight sequence (taking flight, initiation of upwind flight, and source contact) to 119 blend–dosage combinations of the sex pheromone (*Z,Z*- and *Z,E*-7,11-hexadecadienyl acetate). For each behavior the percentage responders was projected as a three-dimensional response surface, after Watkins (1974), and as contour plots. Values on the plots are the percentage of males, of the total tested ($N = 70$ for each treatment), exhibiting a particular behavior. Dosages are milligrams of pheromone applied to rubber septa, and the ratios are percentage *Z,Z* isomer. The thickened 70% contour line approximates the boundary of the peak area of attraction, as determined by hierarchical cluster analysis (see Figure 3).

stage of the response, and in-flight arrestment was not a significant factor in effecting response specificity.

Treatments represented by the response patterns in Figure 2 are identified in Figure 3 within the matrix of blend–dosages tested. Treatments in cluster 1 represent an area of peak attraction, and the boundary separating treatments in

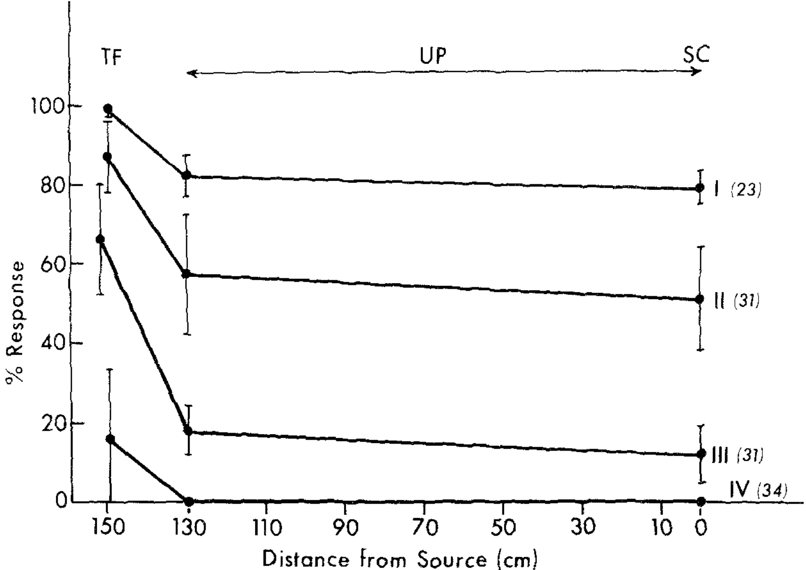


FIG. 2. Response patterns for four clusters (I–IV) of treatments produced by hierarchical cluster analysis. Response values are mean (±SD) for each behavior (as in Figure 1) in the sequence. Values next to the Roman numerals indicate the number of treatments in each cluster.

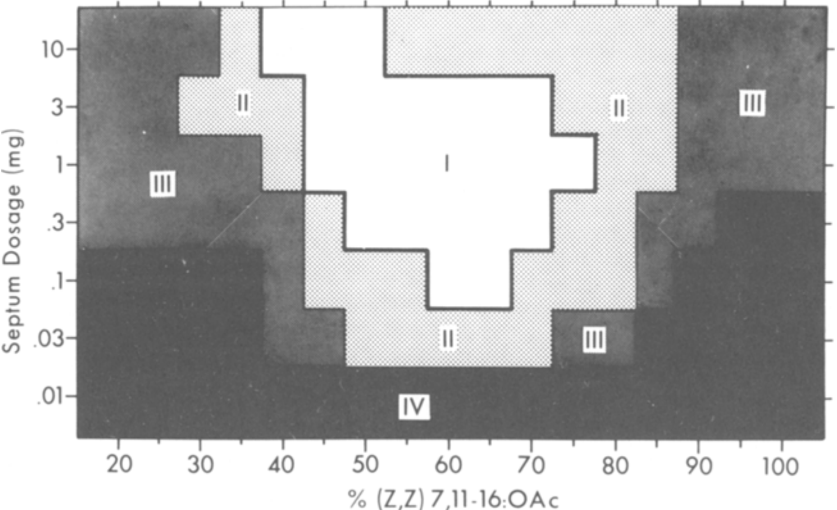


FIG. 3. Pattern of clusters (from Figure 2) within the matrix of 119 combinations of blend and dosage tested. Optimal treatments in the peak area of attraction are designated in the clear area of cluster 1.

cluster 1 from all others represents a threshold region in the blend–dosage matrix for optimal flight response. With respect to the profiles shown in Figure 1, the area of peak attraction is approximated by the 70% contour line. The lowest dosage at which peak response occurred was 0.1 mg, and with blends containing 60–65% *Z,Z* isomer. Increasing the dosage widened the peak area to include blends ranging from 45 to 75% *Z,Z* at 1 mg and 45–70% *Z,Z* at 3 mg. With the highest dosage tested, 10 mg, peak response occurred to blends containing 40–50% *Z,Z*. This represents a significant shift in blend preference over that observed at 0.1 mg. Treatments in the remaining clusters represent successive decreases in response away from the peak region, the result of significant effects on early behaviors, either taking flight or initiation of upwind flight (Figure 2).

## DISCUSSION

In an earlier study (Linn and Roelofs, 1983), male Oriental fruit moths (OFM) responded specifically to blends close to the natural blend and ratio of components (Z8–12:OAc with 6% E8–12:OAc and 3–30% Z8–12:OH). The ratio of *Z/E* isomers was a critical factor in effecting the degree of specificity, since males were more sensitive to changes in the *Z/E* ratio than to changes in the amount of the OH component (Baker et al., 1981; Linn and Roelofs, 1983). A narrow peak area of response centered around the natural 6% *E* blend was bounded by an arrestment threshold (Baker et al., 1981), characterized by treatments containing a higher than normal amount of the *E* isomer (either as a higher than normal ratio at an optimal dosage, or a higher dosage of the normal blend), and an activation threshold for upwind flight characterized by treatments at lower dosages or lower percentage *E* ratios.

Results of the present study agree as well as contrast with those of the earlier study with OFM. In agreement is the observation that the lowest dosage at which peak response occurred with PBW (0.1 mg) was near the natural 61:39 *Z,Z/Z,E* blend (ratios of 60–65% *Z,Z*). Males of both species thus responded optimally to lower dosages of ratios that were closest to the natural blends, supporting the concept that the precise ratio of components emitted by females should be the one that optimally elicits long-distance anemotactic flight (Roelofs, 1978).

The response of PBW males differed, however, from that of OFM in three significant ways. First, there was the absence of an observed arrestment threshold for upwind flight. Regardless of the dosage, over a wide range of ratios representing suboptimal treatments adjacent to the peak area of attraction, male response was most affected in the initial phase of the sequence, that is, in the ability of males to orient and initiate the upwind anemotactic flight. The absence of an observed arrestment effect may, in part, be due to our not testing higher dosages at or beyond the limit of peak attraction. It is possible that at the 30- or 100-mg dosages of blends containing 40–50% *Z,Z* isomer a lowered response

level resulting from an arrestment of upwind flight would occur. Even if this were the case, however, results of the present study show that there was no arrestment of upwind flight as a result of higher percentages of the *Z,Z* isomer, at any dosage tested. The broad area of peak attraction at the higher release rate indicates, in fact, that male upwind flight is not dependent on a precise ratio of components and that arrestment (caused by a higher than normal ratio of isomers, or a higher amount of one isomer) is not a significant factor in controlling response specificity. This is in sharp contrast to OFM, in which response specificity is more closely controlled (as evidenced by a narrower area of peak attraction) and in which both the initiation of upwind flight and its maintenance are affected by blend composition.

A second difference in the response of PBW vs. OFM concerns the size of the peak area of attraction. In a recent study to determine the potential for pheromone resistance in PBW, Haynes et al. (1984) demonstrated that females from a variety of locations in California produced and released a 61:39 ratio of *Z,Z*/*Z,E* isomers. This suggests that females are producing a relatively precise blend, one to which males are most sensitive, as evidenced by the present results and in previous field trials. In contrast to this males exhibited peak levels of response to a significantly wider range of blend–dosage combinations, and therefore a lesser degree of response specificity, than observed with male OFM. The data in Figure 3 show that the greatest range of ratios eliciting peak response occurred with the 1-mg dosage, one that most closely approximates the female release rate (0.14 ng/min for 1 mg on rubber septa vs. a female release rate of 0.1 ng/min *Z,E*, and 0.06 ng/min *Z,Z* isomers; K.F. Haynes and T.C. Baker, unpublished data). The differences in the size of the peak area of attraction, and thus the degree of male response specificity, between OFM and PBW may represent different degrees of adaptation to pressures exerted on the chemical communication system. Two potential sources of selection pressure are intraspecific competition among males in locating females, and the lack of cohabitating species that utilize the same pheromone components.

A third difference in the response of male PBW and OFM was a significant shift in blend preference for PBW away from the region of the natural blend at the highest dosage tested (10 mg) to ones containing 40–50% *Z,Z* isomer. Roelofs (1978) predicted that this shift could explain the fact that in some field trials, ratios other than the natural one were more attractive. Roelofs proposed that higher release rates (due to increasing evaporation of the material, or use of release sources with higher evaporative properties, such as dental wicks as opposed to rubber septa) could result in a broadening of the response or a shift to ratios other than the natural one, depending on the nature of the thresholds controlling response specificity. The observed shift for higher *Z,E*/*Z,Z* ratios in the present study fits with the prediction, but a similar shift to higher *Z,Z*/*Z*,E ratios at higher dosages was not observed. Further field testing with a series of blend ratios over a wide range of dosages would help to clarify this question.

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## Book Review

**The Chemistry of Allelopathy—Biochemical Interactions Among Plants.** Alonzo C. Thompson (ed.). ACS Symposium Series 268. Washington, D.C. American Chemical Society, 470 pp. 1985. U.S.$79.95.

This book is made up of 31 chapters involving 60 authors. As one would expect, this has not resulted in a smoothly flowing narrative. On the other hand, most of the chapters are well-written and very informative. A few of the authors were obviously not aware of some of the literature which was current at the time of the symposium presentation in April 1984.

From a topical standpoint, this book contains: one chapter which gives a brief general overview of allelopathy, one chapter which gives an overview of allelopathy in agriculture, one chapter on the economics of weed control, two chapters on bioassay methods in allelopathic research, eight chapters on allelopathic effects of weeds and/or isolation and identification of allelochemicals from weeds, three chapters on allelopathic effects of crop plants, one chapter on the herbicidal activity of soil microorganisms with emphasis on effects of actinomycetes, one chapter on secondary plant products versus rhizoplane and rhizosphere organisms, one chapter on antimicrobial compounds from plants, one chapter on allelopathic substances from a marine alga, two chapters on allelochemics from aquatic plants, one chapter on naturally occurring substances that inhibit growth of the aquatic plant *Hydrilla verticillata*, one chapter on the role of allelochemicals in the selection of host plants by parasitic angiosperms, two chapters on mechanisms of action of allelochemicals, one chapter on the biosynthesis of phenolic compounds, and four chapters on the synththesis of strigol. The selection of topics for this symposium volume was generally good. However, it does appear a bit unusual to have four chapters on the rather specific topic of strigol synthesis.

For those persons interested in isolating and identifying allelochemicals from plants and microorganisms, this book would be very valuable. Many of the authors have given detailed diagrams of extraction and isolation techniques and many figures and tables of data of spectral characteristics of many compounds. The apparent significance of sesquiterpene lactones, polyacetylenes, and oxygenated fatty acids as allelochemicals in a variety of plants is particularly intriguing to this reviewer. Several of the techniques described in this volume should lead to the identification and quantification of important allelochemicals



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in natural habitats. Such information is essential to studies concerning the fate of the allelopathic compounds after they get out of plants or microorganisms. This is the phase of allelopathy about which the least is known.

In summary, it is obvious that this book is not suitable for a person wishing a general overview of allelopathy. It is an excellent reference, however, for any person seriously involved in research in allelopathy and should be in his or her personal library.

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# INSECT ANTIFEEDANT PROPERTIES OF ANTHRANOIDS FROM THE GENUS *Vismia*

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**Abstract**—Vismiones and ferruginins, representatives of a new class of lypophilic anthranoids from the genus *Vismia* were found to inhibit feeding in larvae of species of *Spodoptera*, *Heliothis*, and in *Locusta migratoria*.

**Key Words**—Vismiones, ferruginins, anthranoids, *Vismia*, antifeedant, *Spodoptera*, *Heliothis*, Lepidoptera, Noctuidae, *Locusta*, Orthoptera, *Acrididae*.

## INTRODUCTION

The role of secondary plant compounds in the defense of plants against insects and fungi has been increasingly recognized in recent years, and a number of investigations have been made to elucidate the chemical basis of plant resistance. Some 10,000 of these compounds are known, with a scattered distribution among higher plants, sometimes with individual compounds common to many plant families or specific to a genus or to a single plant species. A new class of lypophilic anthranoids, vismiones and ferruginins, have been discovered (Marini Bettolo et al., 1978; Delle Monache et al., 1983) in plants of the tribe Vismieae (fam. Guttiferae; subfam. Hypericoideae) which are diffusely distributed in tropical areas. Preliminary tests suggest that these compounds may play a role in the protection of the plant genus *Vismia* against insects. Consequently we have submitted to biological tests, compounds representative of the class.



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## METHODS AND MATERIALS

The structures of the compounds tested are shown in Figure 1. Their effectiveness as feeding deterrents was assessed by presenting them, either on the surface of leaf disks or impregnated in glass fiber disks, to a variety of phytophagous insects.

The insects used were final-instar larvae of the lepidopterans *Spodoptera littoralis*, *S. exempta*, *Heliothis virescens*, and *H. armigera*, and of the locust *Locusta migratoria*. *S. exempta* and *L. migratoria* are oligophagous, their feeding being restricted to species of Gramineae; the other caterpillars are polyphagous. Prior to the test, the insects had access to adequate supplies of food and were at a stage in the instar when feeding is vigorous.

FIG. 1. Structure of compounds used in study. Vismione A (I, R = Ac); deacetylvismione A (I, R = H); vismione B (II, R = H); acetylvismione B (II, R = Ac); vismin (III, $R^1 = R^2 =$ H); ferruginin A (III, $R^1 = R^2 =$ H; isopentenyl chain on C-7); harunganin (III, $R^1 = R^2 =$ H; isopentenyl chain on C-5); ferruginin B (III, $R^1 = R^2 =$ H; isopentenyl chain on C-2); $\gamma$-hydroxyferruginin A (III, $R^1 =$ OH, $R^2 =$ H; isopentenyl chain on C-7); and $\gamma$, $\gamma^1$-dihydroxyferruginin A (III, $R^1 = R^2 =$ OH; isopentenyl chain on C-7).

The compounds were dissolved in 96% ethanol to give a range of concentrations from $2 \times 10^{-5}$ M to $10^{-1}$ M. These solutions were used to prepare treated leaf materials (T); cabbage leaf disks (2.1 cm diam) or wheat leaf blades (3.8 cm long) were immersed in the solutions for 1 min, removed, and dried in a cool air stream. Control leaf material (C) was immersed in 96% ethanol and dried. Glass fiber filter disks (2.1 cm diam) were also used as test substrates since they are readily eaten and avoid the variability inevitably associated with leaf material. They were made palatable by the addition of 200 $\mu$l sucrose solution (0.05 M). When dry, these were used as control disks (C). Treated disks (T) were made by adding 200 $\mu$l of ethanolic solution of test compound to control disks and drying.

Weighed disks, or leaf materials, were presented as a pair (C vs. T) to individual caterpillars in Petri dishes for up to 8 hr so that never more than 50% of any disk was eaten. The locusts were similarly tested, each individual being placed in a clear plastic box $27 \times 15 \times 10$ cm high with a choice of two disks, one treated and one control. The locust tests were run in the dark at 28°C and lasted approximately 3 hr. Both caterpillars and locusts usually consumed 20–45% of the control disk. After removal of the insects, the unconsumed material was reweighed and tests of significance were carried out on weight differences. Leaf material was weighed fresh before and after the test period. During the test period a number of leaf disks were left in pairs in test arenas without insects to allow the effects of evaporative weight loss to be incorporated into the results.

## RESULTS AND DISCUSSIONS

Table 1 allows a comparison to be made, first, of the effectiveness as antifeedants of all the compounds tested at a standard concentration, and second, of the extent to which the different insect species are affected by them. Thus at the $10^{-3}$ M concentration, only acetylvismione B and deacetylvismione A are not significantly deterrent to any of the caterpillars, although the locust was strongly deterred by both of them. Harunganin is the most effective compound, and only *H. armigera* is not significantly affected by it at this concentration. The oligophagous *L. migratoria* and *S. exempta* are the most susceptible species (7 of the compounds gave over 50% feeding inhibition) followed by the polyphagous *H. virescens* (5 compounds over 50%).

Comparison of the antifeedant effect of these compounds on the lepidopterans with that on the orthopteran *L. migratoria* shows that *L. migratoria* is susceptible to a wider range of compounds, and its feeding is reduced to a much greater extent by the compounds than is the case with the lepidopteran larvae. The variation in antifeedant effect of the compounds between the two insect orders is further indicated by the lack of significant relationship between the order of effectiveness of the compounds against *S. exempta* and that against *L.*

TABLE 1. DETERRENT EFFECT [$(C - T)/(C + T)\%$] OF COMPOUNDS IN DUAL CHOICE TEST [CONTROL ($C$) VS. TREATMENT ($T$)] WITH ALL COMPOUNDS TESTED AT $10^{-3}$ M ON GLASS FIBER DISKS

| | Lepidopteran larvae[a] | | | | Orthopteran[a] |
|---|---|---|---|---|---|
| Chemicals ($10^{-3}$ M) | *Spodoptera littoralis* | *Spodoptera exempta* | *Heliothis virescens* | *Heliothis armigera* | *Locusta migratoria* |
| Acetylvismione B | 15.46 b[b] | 18.25 b | 2.95 b | 13.15 b | 100.00 c*** |
| Deacetylvismione A | 29.76 b | 29.29 b | 28.46 b | 18.87 b | 69.22 c*** |
| Vismione A | 51.47 a* | 67.47 b** | 48.86 a* | 25.66 c | 80.14 b*** |
| Vismione B | 36.52 a | 69.69 b** | 58.98 b** | 29.89 a | 100.00 b*** |
| Vismin | 25.06 a | 61.24 b** | 56.56 b** | 39.55 a | 79.22 b*** |
| Ferruginin A | 4.85 b | 54.29 a* | 72.47 c*** | 24.63 a | 29.19 b |
| Harunganin | 48.27 a* | 71.18 c*** | 60.96 c** | 18.94 b | 42.33 a** |
| Ferruginin B | 2.03 c | 9.91 c | 57.76 b** | 29.31 a | 65.50 b*** |
| γ-Hydroxyferruginin A | 27.87 a | 56.36 b** | 47.97 b* | 38.69 a* | 56.42 b*** |
| γ, γ′-Dihydroxy ferruginin A | 5.38 b | 62.64 c** | 34.05 a | 35.55 a | 8.09 b |

[a] $N = 40$ lepidopterans, 10 orthopterans.
[b] Differences between results for the same compounds are shown by letters, so that a and b differ at 0.05 level, a and c at 0.01, and b and c at 0.001. *, **, *** indicate statistical difference from control at 0.05, 0.01, and 0.001 levels, respectively; $t$ test on transformed $\sqrt{(x + 0.5)}$ data.

*migratoria* ($r_s = -0.044$, NS; Spearman rank correlation coefficient), whereas there is significant agreement in the way the chemicals affect the four lepidopteran species ($w = 0.4566$, $P < 0.01$; Kendall coefficient of concordance). However, within the lepidopteran species, there is a significant difference in the magnitude of the response (Kruskal-Wallis $H = 109.51$ $P < 0.001$). There is a significant difference between *S. exempta* and *S. littoralis* ($U = 17$, $P < 0.05$; Mann-Whitney U test) and between *H. virescens* and *H. armigera* ($U = 20$, $P < 0.05$), but not between *S. exempta* and *H. virescens* ($U = 41$, NS) nor between *S. littoralis* and *H. armigera* ($U = 44$, NS).

Thus *L. migratoria* is considerably more susceptible, and its feeding is adversely affected by a wider range of the compounds, than is the case with the lepidopteran larvae, and the compounds which are most effective against *L. migratoria* differ from those most effective against the caterpillars, with acetylvismione B and deacetylvismione A being particularly noteworthy in this respect.

Figure 2 (A–E) examines the effects of different concentrations of test compounds on the lepidopterous larvae. Acetylvismione B (Figure 2A) is equally ineffective for all species at all concentrations tested. Vismione A (Figure 2B) and vismin (Figure 2C) show increasing effectiveness with increasing concentration. With vismione A, there is a threshold level at $10^{-4}$ M, but vismin shows

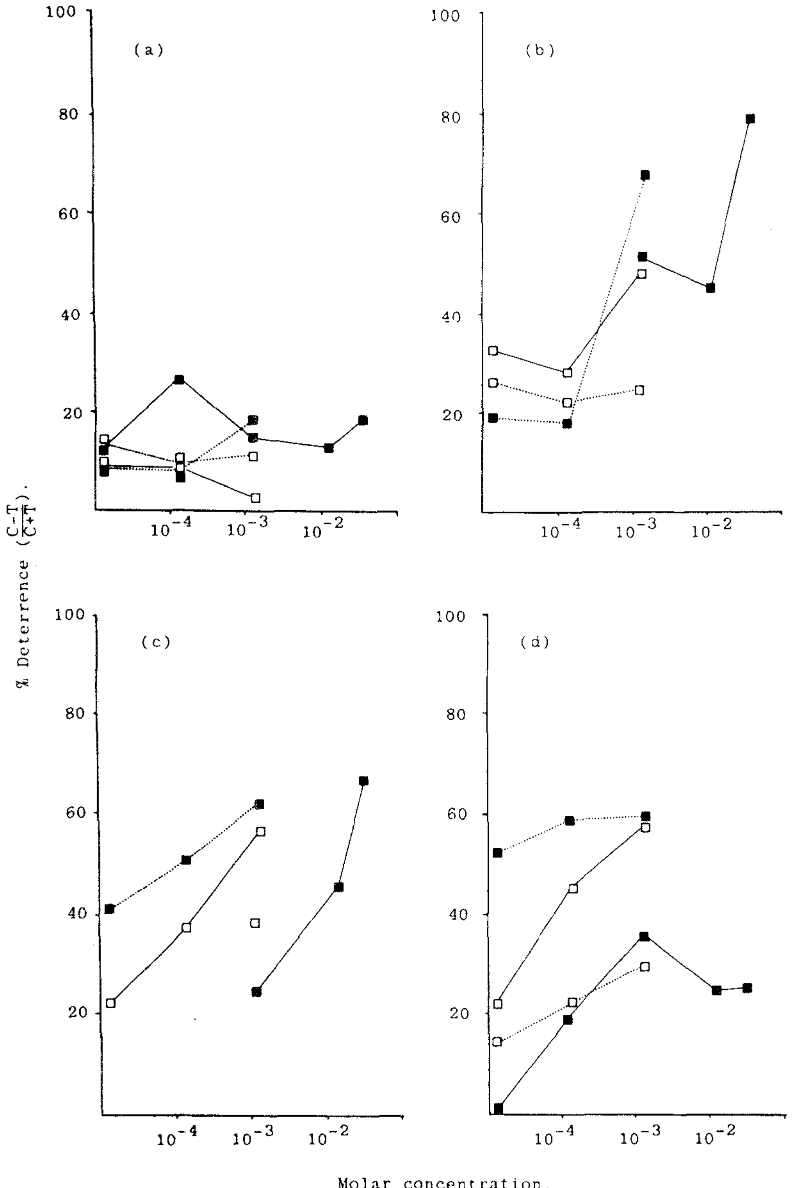


FIG. 2. Effect of concentration on the percent deterrence $[(C - T)/(C + T)\%]$ of compounds against lepidopterous larvae. Compounds tested: (A) acetylvismione B, (B) vismione A, (C) vismin, (D) vismione B, (E) harunganin. *Spodoptera littoralis* ■—■, *S. exempta* ■--■, *Heliothis virescens* □—□, *H. armigera* □--□.

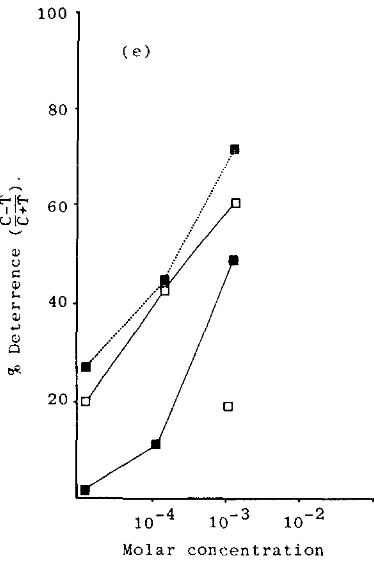


FIG. 2. Continued.

a linear response over the whole range tested. In the case of vismione B (Figure 2D), there is a plateau effect as concentration increases. Harunganin (Figure 2E) shows an increasing dose-dependent response and is the most deterrent compound overall.

Although the use of glass fiber disks avoids the variability associated with plant material, nevertheless leaf disks from host plants are more palatable than glass fiber disks, even when the latter have 0.05 M sucrose added. Consequently, potential feeding deterrents may be less effective on plant material than on glass fiber disks and this should be investigated. Table 2 shows the effectiveness of a few of the compounds applied to leaf disks at the same concentration ($10^{-3}$ M) as that used for Table 1. Comparison of these tables shows that the compounds are much less deterrent when incorporated into fresh plant material taken from palatable host plants than when presented on glass fiber disks. It is of interest that different host plants can modify an insect's response to the compounds. Thus with *S. littoralis*, harunganin is an antifeedant when the host plant is cabbage but is ineffective when the plant is wheat, despite cabbage being preferred to wheat (Blaney and Simmonds, 1983).

Although the range of insects tested here is quite limited, it is nevertheless clear that the two oligophagous species, *S. exempta* and *L. migratoria*, are more susceptible to the test chemicals than are the polyphagous species. Similar results have been obtained with other potentially deterrent compounds of plant origin (Blaney and Simmonds, 1984; Simmonds and Blaney, 1985).

TABLE 2. DETERRENT EFFECT $[(C - T)/(C + T)\%]$ OF COMPOUNDS PRESENTED ON PLANT MATERIAL [CONTROL (C) VS. TREATMENT (T)] IN DUAL-CHOICE TEST[a]

| Chemicals ($10^{-3}$ M) | *Spodoptera littoralis* | | *Spodoptera exempta*, wheat | *Heliothis virescens*, cabbage |
|---|---|---|---|---|
| | Wheat | Cabbage | | |
| Vismione A | 46.74** | 45.01** | 51.47*** | −24.50[b]* |
| Vismin | 8.15[b] | −0.05[b] | −7.37[b] | −3.70[b] |
| Harunganin | 4.96[b] | 34.48** | 44.57** | 58.86*** |
| γ-Hydroxyferrunginin A | 14.23 | 0.98[b] | 8.41[b] | 14.00[b] |

[a] $N = 40$. *, **, *** indicate statistical difference from control at 0.05, 0.01 and 0.001 levels, respectively; $t$ test on transformed $\sqrt{(x + 0.05)}$ data].
[b] Indicates statistical difference from result with glass fiber disks (Table 1).

The differences in responsiveness to antifeedants found here between the lepidopterous larvae and the acridid, emphasize the desirability of testing potential antifeedants against a range of phytophagous insects.

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# ROLE OF PLANT VOLATILES IN RESISTANCE OF SELECTED RICE VARIETIES TO BROWN PLANTHOPPER, *Nilaparvata lugens* (STÅL) (HOMOPTERA: DELPHACIDAE)

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**Abstract**—Rice plant volatiles extracted as steam distillates significantly affected the behavior and biology of the brown planthopper, *Nilaparvata lugens* (Stål). In a multichoice test, more females settled and fed on tillers of a susceptible rice variety "Taichung Native 1" (TN1), sprayed with its own extract or acetone than on TN1 tillers sprayed with the extract of the resistant variety "ARC6650" or "Ptb33." In another test, *N. lugens* females ingested significantly more of a 10% sucrose solution mixed with TN1 steam distillate extract than of plain sucrose solution or that mixed with extracts of resistant varieties. Topical application of the extracts of resistant varieties "Mudgo," "ASD7," "Rathu Heenati," "Babawee," Ptb33, and ARC6650 caused significantly higher mortality of females than did the TN1 extract. Likewise, significantly more first-instar nymphs died when they were caged on susceptible TN1 plants sprayed with the extracts of resistant varieties than on plants sprayed with TN1 extract. The extract of 60-day-old resistant plants was more toxic than the extract of 30-, 45-, or 100-day-old plants. However, toxicity of the extract from susceptible TN1 remained low at all plant growth stages.

**Key Words**—Brown planthopper, *Nilaparvata lugens*, Homoptera, Delphacidae, *Oryza sativa*, plant volatiles, rice, steam distillate.

## INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* (Stål), is a major pest of rice, *Oryza sativa* L., in Asia. Adults and nymphs suck profusely from the phloem tissue of the growing rice plant. Heavy infestations cause complete

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drying or "hopperburn" of the crop. The insect thrives on high-yielding susceptible varieties, but fails to feed, grow, survive, and reproduce adequately on resistant varieties (Pathak et al., 1969). Nonetheless, resistance cannot be traced to any morphological or anatomical peculiarity in the rice plant. Reduced feeding on resistant varieties was, therefore, attributed to either the lack of phagostimulants or to the presence of antifeedants.

Sogawa and Pathak (1970) believed that resistance of "Mudgo" rice plants was possibly due to a somewhat low concentration of amino acids, particularly asparagine which stimulates BPH feeding. On the other hand, Kim et al. (1976) indicated that the *trans*-aconitic acid acted as a BPH antifeedant in the nonhost barnyard grass, *Echinochloa crus-galli* L., a common weed in rice fields. Yoshihara et al. (1979, 1980) reported that soluble silicic acid and oxalic acid in the rice plant acted as BPH sucking inhibitors. But soon it became apparent that silicic acid was a general sucking inhibitor occurring in both susceptible and resistant varieties. Likewise, oxalic acid was found to occur in both resistant and susceptible varieties, although its concentration was slightly higher in some resistant varieties. Shigematsu et al. (1982) identified asparagine as a sucking stimulator and $\beta$-sitosterol as a sucking inhibitor of BPH. However, none of these studies demonstrated that antifeedants extracted from the whole plant occurred principally in the phloem.

Attempts to tap the phloem by various techniques, including the use of laser beams (Kawabe et al., 1980), have not been successful because the vascular bundles in the rice plant are scattered, and so, precise chemical analyses and bioassays of the phloem sap have not been feasible. Even if the presence of antifeedants in the phloem sap of resistant varieties could be demonstrated, it would not relate to other, more vital, aspects of BPH behavior and physiology.

Saxena and Pathak (1979) made systematic studies of BPH–rice plant interactions, particularly behavioral and physiological responses involved in BPH establishment on rice plants. They found that resistant plants were as suitable as susceptible plants in eliciting some responses. The interaction of all the responses determined the overall susceptibility or resistance to the pest. Saxena and Pathak (1979) and Saxena and Puma (1979) also determined the biochemical basis of suitability of rice varieties to BPH. They found that allelochemics and nutritive balance of rice varieties were important in eliciting optimal or suboptimal responses, thereby affecting BPH ability to establish on rice plants. The steam distillate extracts of resistant varieties and of the barnyard grass were repellent and, when applied topically, caused high mortalities even at low doses. In contrast, extracts of susceptible varieties possessed moderate to high attractance and were relatively nontoxic to the insect. Recently, Obata et al. (1983) isolated and identified constituents of the BPH attractant in the Japanese rice cultivar "Nihonbare."

In the present study, we elucidated the role of rice plant volatiles in BPH behavior and biology, particularly on the settling and feeding responses, phago-

stimulation, and survival. The plant growth stage when plant volatiles are best extracted was also determined.

## METHODS AND MATERIALS

BPH biotype 1, which survives on and damages varieties without any resistance genes, was reared on 40- to 60-day-old susceptible "Taichung Native 1" (TN1) rice plants in an insectary at the International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines. The varieties tested were the resistant "Mudgo" (*bph* 1), "ASD7" (*bph* 2), "Rathu Heenati" (*bph* 3), "Babawee" (*bph* 4), "Ptb33" (two resistance genes), "ARC6650" (unidentified resistance genes), and TN1 (no resistance gene). ARC6650 is moderately resistant to all three BPH biotypes in the Philippines (Khush, 1979). The test plants were grown at 20- × 20-cm spacing on 2- × 2-m plots in insect-proof, nylon-screen cages in the field. Seeding was timed such that 30-, 45-, 60-, and 100-day-old plants were available simultaneously for harvesting.

*Extraction of Rice Plant Volatiles.* Rice plant volatiles were obtained as a goup of water-insoluble compounds such as essential oil, particularly terpenoids, low-molecular-weight alcohols, aldehydes, fatty acids, esters, and waxes (Gunther, 1952; Bianchi et al, 1979; Robinson, 1983). Leaves and leaf sheaths of the test varieties were harvested and ground separately with an electric grinder. A 200-g sample of each variety was steam distilled for 4 hr, and about 900 ml distillate was collected and extracted with diethyl ether (300 ml distillate–100 ml diethyl ether) by thoroughly shaking a mixture of the two together in separatory funnel for 5 min. Diethyl ether absorbed the essential oils and other volatiles and settled above the water layer in the funnel. The water layer was discarded, and the extract was pooled in a 500-ml beaker, to which 100 g anhydrous sodium sulfate was added. The resultant mixture was kept inside a fume hood to evaporate excess ether until the volume was about 25 ml. The beaker was covered with aluminum foil and held overnight to allow solium sulfate to absorb traces of water from the extract. The extract was evaporated further to 10 ml and decanted into a preweighed glass vial, which was then covered with perforated aluminum foil and placed inside a desiccator. Ether was evaporated under vacuum, leaving behind a yellow oily odorous residue. The vial was reweighed and the residue dissolved in acetone to desired concentrations.

*Gas Chromatography of Rice Plant Volatiles.* Qualitative analyses of steam distillate extracts were carried out using a 3-m-long column (6.3-mm outer diameter, 2-mm inner diameter), packed with 10% DEGS on 80/100 Gas Chrom Q. The steam distillate extract of each variety was dissolved separately in diethyl ether to a concentration of 20 mg/ml or 20,000 ppm. Six microliters of the solution was then injected into a Varian 2700 chromatograph with hydrogen

flame ionization detector. Operating conditions were: injector temperature 225°C, detector temperature 260°C, column temperature program from 70°C to 200°C at 2°C/min, carrier gas nitrogen, carrier gas flow rate 30 ml/min, attenuation 1/128, range $10^{-12}$ amps/mV. The peaks of each extract were compared by matching and observing the chromatograms on a fluorescent screen. The peaks were not identified but numbered from left to right. The chromatogram of the extract of susceptible variety TN1 was used for comparison with chromatograms of extracts of other varieties.

*Influence of Rice Plant Volatiles on BPH Settling and Feeding Responses* Thirty minutes before exposure to BPH, single tillers of 30-day-old TN1 plants, grown in 6-cm-diameter pots, were sprayed individually with 1 ml of Ptb33, ARC6650, or TN1 extract in acetone at 1 mg extract/tiller using a quick-spray atomizer (Pierce Chemical Co., Rockford, Illinois 61105). Control plants were sprayed with acetone. The pots with treated and control plants were arranged 7 cm apart in a circle in a plastic container (30 cm diameter, 16 cm deep) partly filled with water. The tiller of each test plant was inserted through 1.5-cm-diameter holes bored equidistantly near the periphery of the lid covering the container (Figure 1), and a coded 7-cm filter paper disk was fixed at the base of each tiller. Fifty newly emerged, $CO_2$-anesthetized, brachypterous BPH females were released at the center of the lid and covered with a snug-fitting cylindrical mylar cage (55 cm high, 30.2 cm diameter). Upon revival, the females moved to the plants. Individuals that settled on each plant were counted at 1, 3, 6, 18, 24, and 30 hr after release. As the females fed on their preferred plants and excreted, their honeydew droplets were absorbed on the respective filter paper disks. At the end of the experiment, the filter paper disks were treated with 0.2% ninhydrin–acetone solution and dried in an oven at 40°C for 5 min. The relative size of the bluish amino acid spots on treated filter paper disks gave an estimate of the honeydew excreted by BPH females on treated and control tillers. The experiment was conducted at 27°C and 65–70% relative humidity. Each treatment, including control, was replicated four times.

*Phagostimulation by Rice Plant Volatiles.* The insect was allowed a choice of eight parafilm membrane sachets, seven filled with 10% sucrose solution and a known amount of odorous steam distillate extract of 45-day-old susceptible or resistant plants, and a control sachet filled with sucrose solution only. A 2-$cm^2$ parafilm piece was stretched uniformly over the flared end of the feeding cup. A 2-cm-diameter filter paper disk was then placed on top of the parafilm layer and treated with susceptible or resistant plant extract at 0.1 mg extract/2 $\mu$l acetone per disk. The control was treated with acetone. Treated disks were allowed to stand for 5 min to evaporate acetone, but leaving the odorous extracts. A 0.5-ml aliquot of 10% sucrose solution was then poured on each filter paper disk using a pipet. Another piece of parafilm was stretched over each feeding cup so that the sucrose solution and the extract-treated disk were sandwiched between the parafilm membranes.

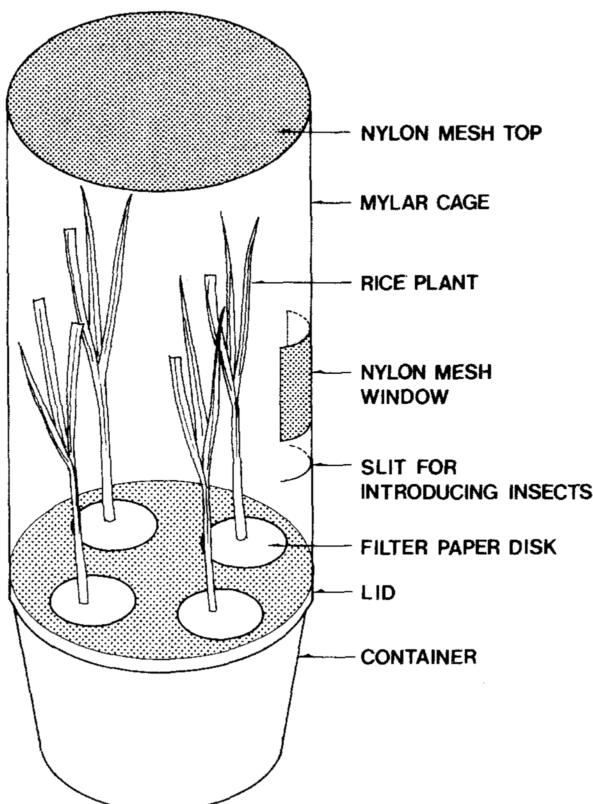


FIG. 1. Mylar cage enclosing rice plants for observing influence of rice plant volatiles on settling and feeding responses of *N. lugens* (biotype 1) females.

The feeding cups and their respective sachets were weighed (W1) and randomly arranged in the feeding chamber, allowing BPH a choice of eight feeding sites, i.e., seven sachets filled with sucrose solution and extract-treated disks and a control with sucrose solution (Figure 2).

The feeding chamber was mounted on a clear, snug-fitting plastic dish partly filled with water for adequate humidity inside the feeding chamber. Twenty newly emerged brachypterous females starved for 3 hr were introduced into the chamber through a small nylon-mesh-covered hole at the center of its lid. The feeding chamber was then kept in an incubator at 26–27°C and 65–70% relative humidity on a yellow cellophane paper. The lid and the side walls of the feeding chamber were covered with black paper permitting yellow light into the chamber from below. After 20 hr, BPH females were removed and the feeding cups were reweighed (W2). The difference (W1 − W2) in the weights gave the quantity of sucrose solution ingested by the females in 20 hr. The experiment was replicated four times in a randomized complete-block design.

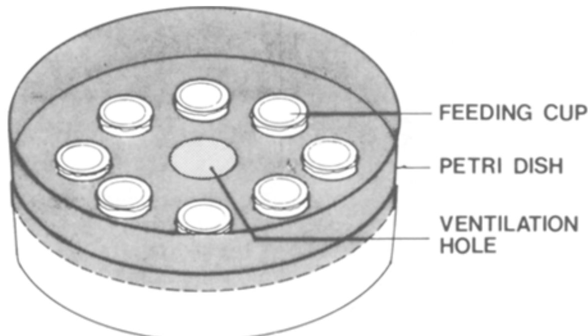


FIG. 2. Feeding chamber for determining phagostimulatory responses of *N. lugens* (biotype 1) females to 10% sucrose solution mixed with steam distillate extract of susceptible TN1 or resistant rice varieties offered in eight parafilm sachets mounted singly on glass feeding cups (3-cm long, 2.45-cm diameter). Feeding chamber was made of a clear plastic Petri dish (14 cm diameter, 2.5 cm high). Feeding chamber bottom had eight peripheral, equidistant, 2.5-cm-diameter holes for feeding cups and a central, nylon-mesh-covered, 2-cm-diameter hole for ventilation. Each feeding cup had one end slightly flared to hold a parafilm sachet and fitted snug and level with any of the eight holes in the feeding chamber.

To adjust the loss in weights of feeding cups, possibly due to evaporation, a blank set without females was similarly arranged and kept randomly with other feeding chambers. The mean weight loss in the blank set gave the weight loss due to evaporation and was used as a correction factor (CF) for determining the net quantity of sucrose solution ingested by BPH females, i.e., (W1 − W2) − CF.

*Toxicity of Rice Plant Volatiles to First-Instar Nymphs.* Steam distillate extracts of 60-day-old test plants were diluted serially in acetone and applied with a camel-hair brush at the rate of 0.01, 0.1, 0.5, or 1 mg extract/ml acetone on the leaf sheaths of 30-day-old TN1 plants potted singly. Controls were treated with acetone. After acetone had evaporated, each treated or control plant was infested with 10 first-instar nymphs and covered with a 30-cm-high, 6-cm-diameter mylar cage. The plants were randomly arranged and kept in an incubator at 26–27°C and 65–70% relative humidity. Insect mortality was recorded after 48 hr and percent corrected mortality was computed using the formula (X − Y/X × 100, where $X$ = percent living nymphs in control, $Y$ = percent living insects in treated group (Abbott, 1925). The experiment was replicated four times in a randomized complete block design.

*Toxicity of Rice Plant Volatiles to Newly Emerged Females.* Steam distillate extracts of 30-, 45-, 60-, and 100-day-old test plants were dissolved separately in acetone to get serial dilutions, 0.1 μl of which gave 5, 10, or 20 μg of the extracts. Newly emerged brachypterous females were lightly anesthetized with

$CO_2$ and treated individually with topical application of the extracts on their dorsum using a Burkard microapplicator. Control individuals were treated with acetone. Treated and control females were caged separately on 20-day-old, potted TN1 plants which were kept at random in an incubator at 26–27°C and 65–70% relative humidity. Insect mortality was recorded after 24 hr and the corrected percent morality was calculated using Abbott's formula. The experiment was replicated four times in a randomized complete block design with 10 females per replication.

All data were subjected to analysis of variance, and the means were compared using Duncan's (1951) multiple-range test at the $P = 0.05$ level.

## RESULTS

*Extraction of Rice Plant Volatiles.* The susceptible TN1 plants yielded relatively less extract (56–75 mg/200 g fresh leaf sheaths) than the resistant varieties (Table 1). Optimum yields of the steam distillate extract were obtained from 45- to 60-day-old plants in most of the varieties.

The steam distillate extract had two distinct layers after evaporation of diethyl ether: a whitish waxy layer below a yellowish oily layer. The proportion of these two layers seemed to vary from variety to variety and also with plant parts. The whitish waxy layer seemed to be greater in leaf sheath than in leaf samples. The oily layer was more readily soluble in acetone than was the waxy layer. However, both layers dissolved readily in diethyl ether.

*Gas Chromatography of Rice Plant Volatiles.* Thirty-four distinct peaks were recorded (Figure 3), two very prominent peaks—peak number 10 with

TABLE 1. EFFECT OF PLANT AGE ON YIELD OF STEAM DISTILLATE EXTRACTS OF RICE VARIETIES SUSCEPTIBLE OR RESISTANT TO *N. lugens* (BIOTYPE 1)

| | Yield of extract (mg/200 g fresh weight) in plants at given days after seeding | | | |
|---|---|---|---|---|
| Variety | 30[a] | 45[a] | 60[b] | 100[a] |
| Mudgo | 78.0 | 103.6 | 186.8 | 76.0 |
| ASD7 | 76.2 | 177.7 | 109.1 | 87.8 |
| Rathu Heenati | 106.0 | 97.6 | 147.5 | 52.4 |
| Babawee | 147.4 | 142.7 | 137.6 | 80.8 |
| Ptb33 | 82.4 | 84.2 | 149.7 | 65.8 |
| ARC6650 | 70.5 | 144.0 | 132.9 | 68.1 |
| TN1 | 56.1 | 72.9 | 65.9 | 75.3 |

[a] Average of two replications.
[b] Average of four replications.

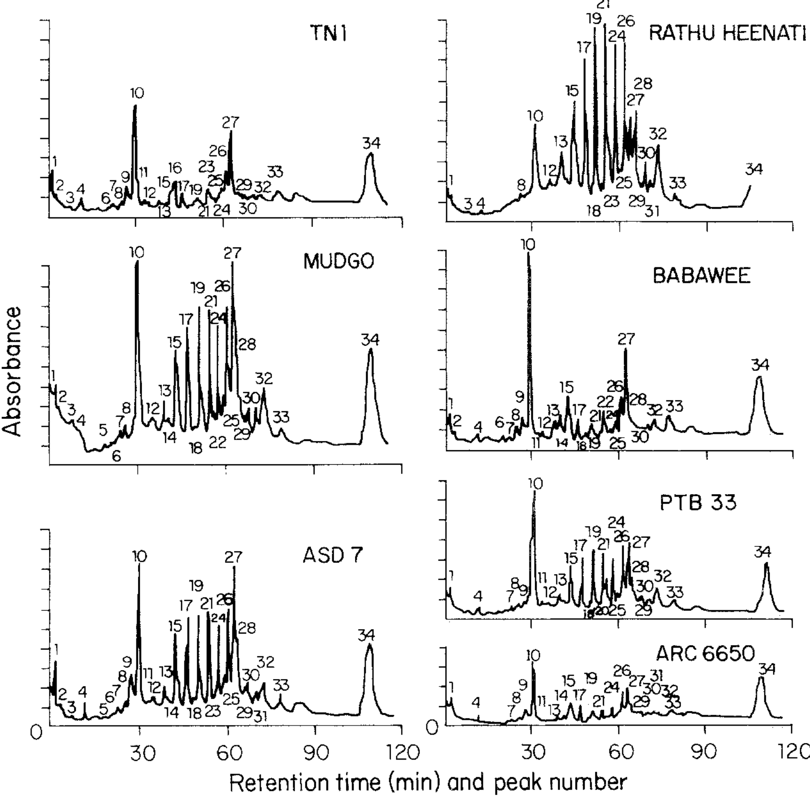


FIG. 3 Gas chromatograms of volatiles of leaf sheaths of rice varieties susceptible (TN1) and resistant to *N. lugens* (biotype 1).

retention time (RT) of 30 min and peak number 27 with RT of 59.1 min—were observed in all varieties. Many peaks were common to all varieties but showed variation in absorbance. However, there were also some unique peaks for certain varieties. For example, peak number 16 with RT of 43.6 min was recorded in TN1, while peak number 28 with RT of 60 min appeared only in resistant varieties but not in TN1. Peak number 15 with RT of 41.4 min was more prominent in resistant varieties than in TN1. The more conspicuous differences among the resistant varieties were variations in absorbance of specific peaks. For example, peak number 28, which appeared only in resistant varieties, was more prominent in Rathu Heenati than in other resistant varieties. Peak number 14, which appeared in Mudgo, ASD7, Babawee, and ARC6650, was more distinct in Babawee than in the other varieties.

*Influence of Rice Plant Volatiles on Settling Responses.* In the choice test, BPH females settled uniformly on TN1 plants sprayed with acetone or with the

TABLE 2. SETTLING RESPONSE OF *N. lugens* (BIOTYPE 1) FEMALES ON TN1 PLANTS SPRAYED WITH STEAM DISTILLATE EXTRACTS OF RESISTANT AND SUSCEPTIBLE RICE VARIETIES AT 1, 3, 6, 18, 24, AND 30 HR AFTER RELEASE IN FREE-COICE TEST[a]

| | Individuals settled (%) on plants at given hr after release[b] | | | | | |
|---|---|---|---|---|---|---|
| Variety | 1 | 3 | 6 | 18 | 24 | 30 |
| Ptb33 | 14 a | 16 a | 9 b | 8 b | 10 bc | 8 b |
| ARC6650 | 15 a | 14 a | 17 ab | 7 b | 5 c | 8 b |
| TN1 | 22 a | 25 a | 14 ab | 15 ab | 17 ab | 17 b |
| Control (acetone-sprayed) | 8 a | 12 a | 25 a | 31 a | 34 a | 40 a |

[a]In a column, means followed by a common letter are not significantly different by Duncan's (1951) multiple-range test at the $P = 0.05$ level.
[b]Averages of four replications, each replication had 50 newly emerged females. Percentages calculated on total number of females released in the test chamber; remaining individuals on the surface, below plants.

extracts of the resistant Ptb33 and ARC6650 and the susceptible TN1 rice varieties during the first 3 hr after their introduction into the test chamber. But 6 hr after introduction, there were significantly less females on TN1 plants sprayed with Ptb33 extract than on acetone-treated plants (Table 2); however, the number of the individuals on plants sprayed with TN1 or ARC6650 extract was not different from the control.

Even after 18, 24, and 30 hr, significantly less females were recorded on TN1 plants sprayed with the extracts of the resistant variety Ptb33 or ARC6650 than on control plants. The number of individuals on TN1 extract-treated plants was statistically the same as on control plants at 18 and 24 hr after introduction.

A visual comparison of honeydew spots on filter paper disks showed that BPH females fed more and consequently excreted more on control and TN1 extract-treated plants than on those treated with extract of Ptb33 or ARC6650 extract (Figure 4).

*Phagostimulation of Rice Plant Volatiles.* In the choice test, incorporation of the extract of the susceptible TN1 variety into 10% sucrose solution stimulated significantly greater intake than that of plain sucrose solution or that with the extract of the resistant varieties Mudgo, Rathu Heenati, Babawee, Ptb33, or ARC6650 (Table 3). The resistant variety ASD7 was an exception, and BPH females fed as well on sucrose solution with ASD7 extract as that with TN1 extract. The extracts of Ptb33 and Rathu Heenati caused maximum inhibition of intake of sucrose solution.

*Toxicity of Rice Plant Volatiles to First-Instar Nymphs.* Significantly more first-instar nymphs died on TN1 plants treated with 1 mg extract/plant of the

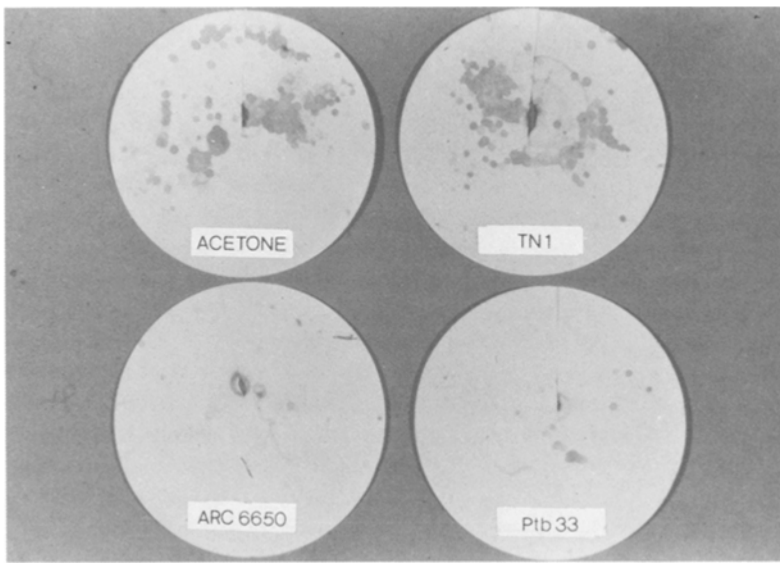


FIG. 4. Filter paper disks on which honeydew of *N. lugens* (biotype 1) females was collected when they fed on susceptible TN1 plants sprayed with steam distillate extract of resistant ARC6650, Ptb33, or susceptible TN1 rice varieties. Control plants were sprayed with acetone. Dark spots on ninhydrin-treated filter paper disks indicate the amount of honeydew excreted by females on treated rice plants.

TABLE 3. PHAGOSTIMULATORY RESPONSE OF *N. lugens* (BIOTYPE 1) FEMALES TO 10% SUCROSE SOLUTION INCORPORATED WITH STEAM DISTILLATE EXTRACTS OF RESISTANT AND SUSCEPTIBLE RICE VARIETIES OFFERED IN PARAFILM SACHETS IN A FREE-CHOICE TEST[a]

| Variety | Quantity of sucrose solution ingested[b] (mg/20 females/20 hr) |
|---|---|
| Mudgo | 4.55 bcd |
| ASD7 | 12.50 a |
| Rathu Heenati | 2.35 cd |
| Babawee | 6.53 bc |
| Ptb33 | 0.66 d |
| ARC6650 | 7.93 b |
| TN1 (susceptible check) | 12.04 a |
| Control[c] | 6.31 bc |

[a] Each parafilm sachet had 0.1 mg extract incorporated in 0.5 ml of 10% sucrose solution.
[b] Means followed by a common letter are not significantly different by Duncan's (1951) multiple-range test at the $P = 0.05$ level; averages of four replications.
[c] Sucrose solution only.

resistant varieties than on control plants treated with the extract of susceptible TN1 (Table 4). Even at 0.5 and 0.1 mg extract/plant, more nymphs died on plants treated with extracts of some resistant varieties than on those treated with TN1 extract. However, nymphal mortality on plants treated with 0.01 mg extract of susceptible TN1 and of resistant varieties did not significantly differ.

BPH nymphs did not exhibit symptoms of toxicological trauma, but most simply rejected the treated plants and settled on the mylar cages. Thus, death resulted partly from starvation and exhaustion in addition to any innate toxicity of the volatile principals in the extract.

*Toxicity of Rice Plant Volatiles to Newly Emerged Females.* Topical application of steam distillate extracts from rice plants reduced survival of BPH females. At a dose of 5 μg extract/female, the steam distillate extracts of susceptible and resistant varieties did not differ in toxicity. Insect mortality increased progressively with increased doses of the extracts of both susceptible and resistant varieties, but higher doses of extracts of the resistant varieties were significantly more toxic than higher doses of extract of susceptible TN1 (Table 5). Ptb33 extract was most toxic to BPH females.

Plant age influenced the toxicity of the extracts in resistant varieties (Table 6). At a dose of 20 μg extract/female, the extract from 30-day-old resistant plants was less toxic than that from 45-, 60-, and 100-day-old plants. The extracts from 45- and 100-day-old plants were comparable in toxicity but were significantly less toxic than those from 60-day-old plants. Thus, toxicity in resistant rice plants increased up to panicle initiation, but decreased at maturity. However, toxicity of the extract of susceptible TN1 plants was low at all plant growth stages.

TABLE 4. MORTALITY OF FIRST-INSTAR *N. lugens* (BIOTYPE 1) NYMPHS ON TN1 RICE PLANTS[a] TREATED WITH STEAM DISTILLATE EXTRACTS OF RESISTANT AND SUSCEPTIBLE RICE VARIETIES[b]

| | Nymphal mortality (%) extract-treated plants | | | |
|---|---|---|---|---|
| Variety | 0.01 mg | 0.1 mg | 0.5 mg | 1.0 mg |
| Mudgo | 0 b | 8 abc | 10 cd | 70 a |
| ASD7 | 13 a | 11 abc | 16 bc | 61 a |
| Rathu Heenati | 5 ab | 13 ab | 21 abc | 73 a |
| Babawee | 5 ab | 2 c | 24 ab | 71 a |
| Ptb33 | 8 ab | 18 a | 32 a | 70 a |
| ARC6650 | 8 ab | 16 a | 35 a | 52 a |
| TN1 (susceptible check) | 2 ab | 5 bc | 0 d | 40 b |

[a] Thirty-day-old plants.
[b] In a column, means followd by a common letter are not significantly different by Duncan's (1951) multiple-range test at the $P = 0.05$ level); averages of four replications, 10 nymphs per replication.

TABLE 5. MORTALITY OF *N. lugens* (BIOTYPE 1) FEMALES TOPICALLY TREATED WITH STEAM DISTILLATE EXTRACTS OF RESISTANT AND SUSCEPTIBLE RICE VARIETIES[a]

| | Mortality (%) at given dose/female | | |
|---|---|---|---|
| Variety | 5 μg | 10 μg | 20 μg |
| Mudgo | 7 a | 37 ab | 59 abc |
| ASD7 | 6 a | 35 ab | 56 bc |
| Rathu Heenati | 5 a | 30 ab | 64 ab |
| Babawee | 5 a | 34 ab | 58 bc |
| Ptb33 | 7 a | 41 a | 71 a |
| ARC6650 | 4 a | 27 b | 52 c |
| TN1 (susceptible check) | 3 a | 15 c | 32 d |

[a] In a column, means followed by a common letter are not significantly different by Duncan's (1951) multiple-range test at the $P = 0.05$ level; averages of four replications, 10 females per replication.

TABLE 6. INFLUENCE OF AGE OF RICE PLANT ON RELATIVE TOXICITY OF STEAM DISTILLATE EXTRACT TO *N. lugens* (BIOTYPE 1) FEMALES[a]

| Plant age (days after seeding) | Mortality (%) at given dose/female | | |
|---|---|---|---|
| | 5 μg | 10 μg | 20 μg |
| 30 | 5 a | 24 b | 38 c |
| 45 | 5 a | 31 b | 55 b |
| 60 | 5 a | 41 a | 77 a |
| 100 | 6 a | 29 b | 53 b |

[a] In a column, means followed by a common letter are not significantly different by Duncan's (1951) multiple-range test at the $P = 0.05$ level; averages of four replications, 10 females per replication.

BPH females showed no overt symptoms of toxicity, such as twitching of body parts, but simply became weak and dropped to the base of rice plants. In some cases, they died while clinging to the plant. Many treated females wandered inside the cages.

## DISCUSSION

Plants serve not only as food but also as a microhabitat, shelter, and protection for the phytophagous insects (Dethier, 1970). This implies that the bionomics of an insect pest would largely depend on the host plant's physical and chemical makeup. According to Chapman and Bernays (1977), plant chemical factors are very important in plant–insect relationships. The results of the present

study have elucidated the role of rice plant volatiles as possible "allelochemics" (Whittaker and Feeney, 1971) involved in BPH–rice plant interactions.

The low settling response of BPH females on tillers of the susceptible TN1 plants treated with the steam distillate extracts of the resistant varieties indicated that the treatment conferred resistance at least temporarily. The low amount of honeydew excreted by BPH females on the tillers of such TN1 plants confirmed that the insect was unable to settle down for sustained feeding. Nymphs caged on similarly treated TN1 plants were unable to settle on them and suffered high mortality. The observed restlessness of BPH nymphs and adults on resistant varieties can thus be attributed to the emanating volatile compounds which are deleterious to the insect. This was confirmed by topical application of the steam distillate extracts of the resistant rice varieties on BPH females. The low mortality of BPH females treated with TN1 extract indicated that some "allomones" or defense chemicals may also occur in the susceptible variety, but their concentration is lower than that in resistant varieties. Alternatively, the resistant varieties may possess defense chemicals which are absent altogether in susceptible ones.

The increased or decreased toxicity of the steam distillate extract of resistant rice plants may be accounted for as suggested by McKey (1979). A low concentration of allelochemics in the young growing plant tissue may be due to a dilution effect from other compounds being synthesized in a particular plant tissue. On the other hand, a decrease in concentration of defense chemicals in maturing or senescing plants may be due to rapid loss by volatilization, leaching by rainfall (Flück, 1963), autoxidative polymerization (Goldstein and Swain, 1963), and possibly low synthesis.

Greater ingestion of 10% sucrose solution incorporated in the steam distillate extract of the susceptible TN1 or the resistant ASD7 rice plants than of sucrose solution alone suggests that the extracts of those plants had a characteristic odor that stimulated BPH feeding. The fact that steam distillate extracts of resistant varieties Mudgo, Rathu Heenati, Babawee, Ptb33, and ARC6650 did not stimulate as much intake of sucrose solution as did the TN1 extract indicated that those resistant varieties may possess some additional chemical factors which nullified feeding stimulation. Alternatively, the feeding stimulant factors in Mudgo, Rahtu Heenati, Babawee, Ptb33, and ARC6650 varieties may be so concentrated as to become inhibitory or even toxic to BPH. Reese (1979) pointed out that a compound that is a requirement at one concentration may be highly toxic at a 10-fold concentration.

The exact identity of allelochemics in the test rice varieties is not known. However, with the methodology that we used, a large group of compounds, such as essential oils, particularly terpenoids, low-molecular-weight alcohols, aldehydes, fatty acids, esters, waxes, etc., were obtained (Gunther, 1952; Bianchi et al., 1979; Robinson, 1983). According to Bernays (1982), the insects are relatively sensitive to certain chemical classes, such as the terpenoids. Recently,

Obata et al. (1983) indentified a mixture of fourteen esters, seven carbonyl compounds, five alcohols, and one isocyanurate in the volatile attractant fraction of BPH-susceptible Jananese rice cultivar Nihonbare.

Most plant volatile compounds are sparingly soluble in water and are not likely to be translocated in the vascular bundles (McKey, 1979). However, their odoriferous and volatile nature makes them exert a strong influence on the total chemical environment of the rice plant and hence is of ecological significance in determining the susceptibility or resistance of rice plants to BPH. Saxena (1978) reported a wide range of effects of rice plant steam distillates on the behavior and physiology of the striped stem borer, *Chilo Suppressalis* (Walker) and their implications for susceptibility or resistance to the pest.

Whittaker (1970) reported that to be effective, the defense chemicals must be relatively toxic. Further, to make them available to herbivores, the plant must be to some degree leaky and open to some loss of materials through its surfaces. In the same manner, the rice plant volatiles may be able to interact with BPH, even though the volatile compounds may not occur at the feeding site. Volatile compounds from resistant host plants may penetrate the insect body through the cuticle or spiracles during feeding and respiration. Cuticular penetration of plant volatiles is well documented in insects. For example, nicotine applied to one anterior wing of the American cockroach, *Periplaneta americana* (L.), was detected in various tissues of the insect (Ebeling 1964). BPH nymphs, particularly young instars, having a vestiture of poorly chitinized cuticle and relatively larger surface area because of smaller size, would therefore be vulnerable to volatile compounds of resistant rice plants, while attempting to feed on them.

The possibility of occurrence of BPH feeding inhibitors in resistant rice plants has also been suggested. Yoshihara et al. (1979, 1980) reported soluble silicic acid and oxalic acid as sucking inhibitors in rice plants: both occur in susceptible and resistant rice varieties, but oxalic acid is relatively higher in the resistant than in the susceptible varieties. Although both these acids are water soluble, Their occurrence in the phloem sap has not yet been demonstrated. Silica, which is the elemental form of the silicic acid and occurs in the soil, is more likely to be transported through the xylem vessels. On the other hand, oxalic acid, which is a product of plant metabolism, is highly toxic even to the plant tissue and, therefore, less likely to occur freely in the phloem. In plants, oxalic acid normally accumulates in the tonoplasm of vacuoles of the cells (Buvat, 1969).

Bioassays of the steam distillate extracts of varieties susceptible or resistant to BPH seem to support Fraenkel's (1959, 1969) view that plant volatiles form a vital part of the total phagostimulation/repellent flavor of the plant, and potential nutritiveness of the plant is a complementary factor.

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# COMPARED BEHAVIORAL RESPONSES OF MALE *Drosophila melanogaster* (Canton S) TO NATURAL AND SYNTHETIC APHRODISIACS

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**Abstract**—Cuticular aphrodisiacs from *D. melanogaster* females were further characterized and the male response specificity towards such natural and synthetic unsaturated hydrocarbons was investigated. The behavioral activity seems to be correlated with some chain-length requirement and double-bond position; at least one double bond in position 7 seems necessary. This position is more abundant among natural monoenes, and among dienes which also bear a second double bond in position 11, whatever the chain length. Bioassays of the synthetic (Z,Z)-7,11-heptacosadiene yielded a dose–response curve close to that of the natural mixture of heptacosadienes in which the 7-11 isomer is predominant. This female specific 7,11 heptacosadiene appears to be the most potent aphrodisiac for males of the species. Its threshold is lower than that of both 7,11-nonacosadiene and 7-pentacosene which might also play a role in sex and species recognition.

**Key Words**—*Drosophila melanogaster*, *Diptera*, *Drosophilidae*, aphrodisiac pheromone, recognition pattern, cuticular hydrocarbons, *heptacosadienes*, mass spectrometry.

## INTRODUCTION

Chemical communication is a common means among insects to control sequences of precopulatory behavior and orient them towards a partner of the appropriate sex and species. In this respect, hydrocarbons from the cuticle of many insects have been found to be behaviorally active (Howard and Blomquist,

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1982). In *Drosophila melanogaster*, the existence of behaviorally active substances has been suggested for some years by various authors (for a review, see Ehrman, 1978). In recent work, female compounds were shown to induce male precopulatory behavior in the strain Canton S (Venard and Jallon, 1980; Tompkins et al., 1980).

The isolation of female active compounds required a quantitative behavioral test. For this, Jallon and Hotta (1979) supposed that the intensity of male courtship reflected the intensity of stimuli produced by females. They chose to measure the average value of cumulative time of characteristic wing vibrations in a number of standardized males. This measurement was used by Antony and Jallon (1982) in bioassays designed to test candidate compounds from fly extracts obtained by cuticular washes of mature females (3–4 days) with hexane. Bioassays demonstrated that the induction of male wing vibrations was associated with female cuticular compounds but not with male ones. Such extracts were then analyzed with gas–liquid chromatography coupled with mass spectrometry (GC-MS) after partial purification by a combination of thin-layer chromatography and preparative gas chromatography. Active compounds were found to be long-chain unsaturated hydrocarbons (25–29 carbons), either dienes or monoenes. Sexual dimorphism was clear, as only female *D. melanogaster* produced dienes. It was shown that for a dose equivalent to that borne by a single female, only the heptacosadiene fraction was behaviorally active (Antony and Jallon, 1982).

The data reported here deal with the characterization of unsaturated hydrocarbons from females and an analysis of the male response towards natural and synthetic compounds.

## MATERIALS AND METHODS

Wild-type Canton S strain flies were bred at 25°C with a 12/12 dark–light cycle. Sexes were separated a few hours after eclosion under a light ether anesthesia. Males and females were held separately until maturity.

Cuticular hydrocarbons were obtained from extracts resulting from cuticular washes of flies of given sexes and ages. Macroextracts were obtained with 250 flies bathed in 1.5 ml hexane for ca. 2 min and shaken for 1 min in a vortex. Microextracts were obtained by submerging a single fly in 50 $\mu$l hexane for 2 min and shaking for 1 min. Both extracts were the same as those described by Antony and Jallon (1982). Purified fractions were quantified using standard commercial alkanes.

For bioassays, a given amount of hydrocarbon was deposited with a microcap on a "dummy" male consisting of a freshly killed male thoroughly washed with hexane. Hexane was evaporated for 2 min, and the dummy was introduced into an observation chamber together with a test male. Cumulated wing vibra-

tion times of test males ($\overline{\Sigma T_N}$) were measured for 5 min as described by Jallon and Hotta (1979).

For ozonolysis, ozone was allowed to flow 1 min into hexane solutions of unsaturated dienes or monoenes separated according to their chain lengths. The ozonides were injected directly into the GC to release aldehydes. The chain lengths of the resulting aldehydes were determined according to their comigration in gas chromatrography (GC) with standard aldehydes or by coupled gas chromatography–mass spectrometry (GC-MS).

For methoxymercuration–demercuration, the procedure used was the same as that one previously described by Pechine et al. (1985).

(*Z*,*Z*)-7,11-heptacosadiene and a few monoenes [(*Z*)-7-tricosene, (*Z*)-7-pentacosene, (*Z*)-9-tricosene, (*Z*) 9-pentacosene, and (*Z*)-9-heptacosene] were synthesized (Davis and Carlson, manuscript in preparation; Carlson et. al., 1974). The synthetic (*Z*,*Z*)-7,11-heptacosadiene contained 2.5% of the geometric isomers *Z*,*E* and *E*,*Z*, as measured by gas chromatography.

## RESULTS

*Further Characterization of Female Cuticular Unsaturated Hydrocarbons.* Antony and Jallon (1982) described hydrocarbons present in the cuticles of fe-

TABLE 1. AMOUNTS OF UNSATURATED CUTICULAR HYDROCARBONS BORNE BY MATURE FEMALE AND MALE *D. melanogaster* CANTON S STRAIN INDIVIDUALS (4 DAYS OLD, IN ng/fly)[a]

| | | | Females (30) | Males (30) |
|---|---|---|---|---|
| 23C | diene | | | |
| | monoenes | A | ± | ± |
| | | B | 52.5 (±7.5) | 423 (±7.8) |
| | | C | | 24.8 (±2.6) |
| 25 C | diene | | 36 (±4.5) | |
| | monoenes | A | 106.5 (±12) | ± |
| | | B | 108 (±9) | 96 (±11.5) |
| | | C | 7.5 (±1.5) | |
| 27 C | diene | | 420 (±25.5) | |
| | monoenes | A | | |
| | | B | 70.5 (±9) | ± |
| | | C | | |
| 29 C | diene | | 192 (±19.5) | |
| | monoenes | A | | |
| | | B | | ± |
| | | C | | |

[a] The sign ± represents trace.

male (26 components) and male (15 components) *D. melanogaster* Canton S. They also presented quantification of extracts of large pools of flies 3–5 days old. Jallon (1984) recently compared the unsaturated components of individual mature 4-day-old flies of both sexes (Table 1). Although a few small quantitative differences concerning major compounds in either sex were apparent in individuals as compared with pools, the clear sexual dimorphism of cuticular hydrocarbons was confirmed. It appeared that interindividual variability is relatively low, always less then 15%. Some of the minor components evidenced in pools were not seen in these microextracts, but the major component of female cuticles, absent in male cuticles, was the same heptacosadiene; its GC-MS spectrum is shown in Figure 1A.

Several isomers for female monoenes and dienes were shown by GC-MS analysis. The set of individual chromatograms clearly showed several peaks for tricosene, pentacosene, and heptacosene and one peak for pentacosadiene, heptacosadiene, and nonacosadiene. It was then necessary to try to determine the positions of double bonds in these isomers. These positions were determined using two chemical methods, ozonolysis and methoxymercuration–demercuration, followed by the analysis of resulting products with GC and GC-MS. Using the latter method to study the monoenes of *D. melanogaster* males and *D. simulans* males and females, Pechine et al. (1985) have shown that it is not necessary to separate the various monoenes and dienes.

For female monoenes, methoxymercuration–demercuration of total extracts followed by a GC-MS analysis with chemical ionization showed clearly that at least two positional isomers were present at each chain length of 23, 25, and 27 carbons. A quantitative estimate for isomers 7-8 (B) and 9-10 (A) deduced from ratios of mass fragmentation peak heights is shown in Table 2 and is compared with data calculated directly from GC peak areas. The two pentacosenes are present in similar amounts, whereas 7-tricosene and 7-heptacosene are very dominant. The ozonolysis of isolated mixtures of monoenes with either 23, 25, and 27 carbons yielded fragments which comigrated by GC with aldehydes comprising chain lengths with 14/16, 16/18, and 18/20 carbons, respectively. The main aldehydes always corresponded to position 7 (isomer B), but position 9 was also present (isomer A) in lower amounts, as already described in male hydrocarbons. Both methods suggest the existence of small amounts of other positional isomers, probably at carbons 5–11.

The determination of double bonds in dienes was performed with similar chemical methods. Methoxymercuration–demercuration led to dimethoxy dienes which, when analyzed by GC-MS, could be interpreted more easily than those of monomethoxy derivatives. An example is shown for heptacosadienes (Figure 1B). Four possible dimethoxy derivatives may be formed, depending on the position of the methoxy groups. These can be on either carbon supporting the double bond, leading to 8 expected cleavages (aa′ to dd′, Figure 1B). Di-

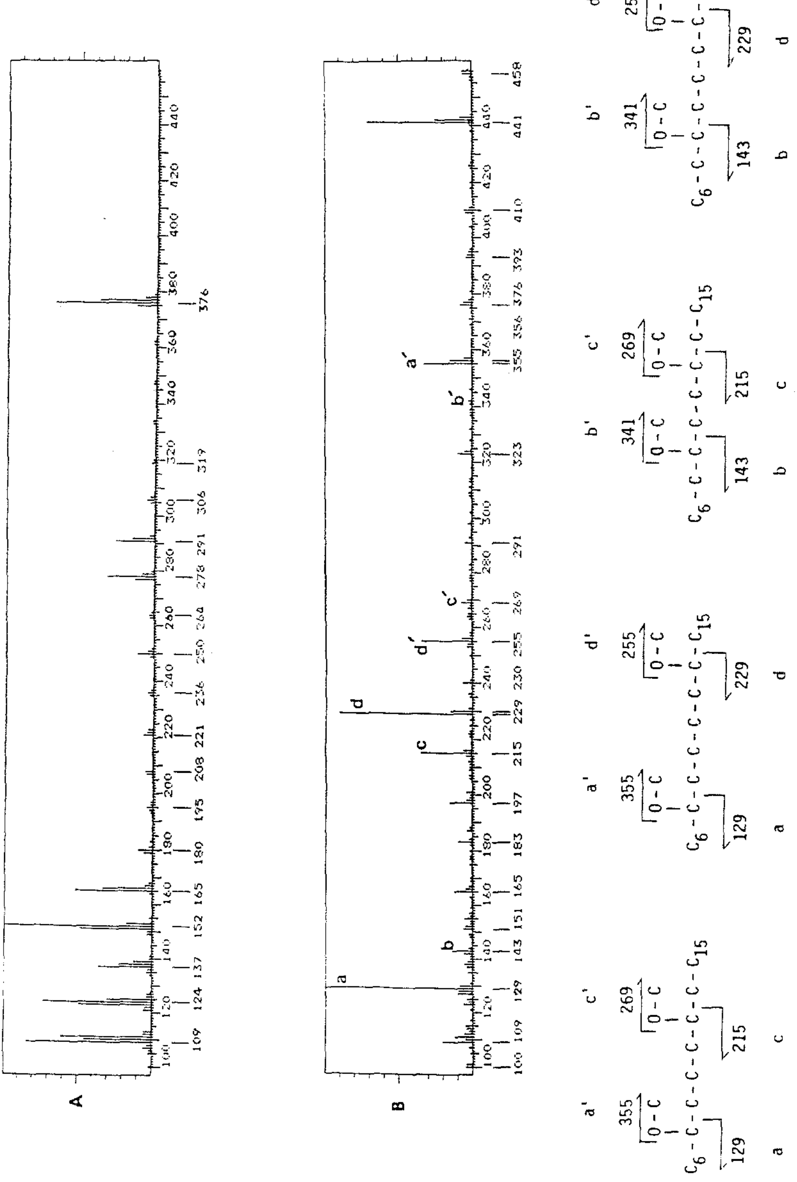


FIG. 1: Mass spectra of the heptacosadiene fraction of female *D. melanogaster*. (A) Natural, chemical ionization with methane. (B) Dimethoxy derivatives, chemical ionization with ammonia.

TABLE 2. DOUBLE-BOND POSITIONS FOR MONOENE ISOMERS (A, B) AND DIENES DETERMINED BY METHOXYMERCURATION–DEMERCURATION AND OZONOLYSIS[a]

| | | Position of double bonds | GC areas (%) | Methoxymercuration–demercuration (%) | Ozonolysis |
|---|---|---|---|---|---|
| Monoenes | | | | | |
| 23 C | A | 9 | 10 | 10 | minor |
| | B | 7 | 90 | 90 | major |
| 25 C | A | 9 | 45 | 40 | minor |
| | B | 7 | 55 | 60 | major |
| 27 C | A | 9 | 26 | 15 | minor |
| | B | 7 | 74 | 85 | major |
| Dienes | | | | | |
| 25 C | | 7,11 | | | |
| 27 C | | 7,11 | | | |
| 29 C | | 7,11 | | | |

[a] The relative abundances of the various positional isomers are indicated either as percentages or levels (number of crosses).

methoxy derivatives had $OCH_3$ groups preferentially located on the C-27 diene backbone as far from each other as possible. The fragmentation pattern peaks aa′ and bb′ are clearly associated with fragmentations adjacent to carbons 7 and 8, respectively; peaks cc′ and dd′ correspond to fragmentation adjacent to carbons 11 and 12. Ozonolysis of isolated dienes with 25, 27, and 29 carbons always led to the following major fragments: a-aldehydes with 14, 16, and 18 carbons were found for hydrocarbons with, respectively, 25, 27, and 29 carbons, indicating a common position 11-12 for one of the double bonds; b-heptanal was obtained in all chain lengths, suggesting that the other double bond was always present in position 7-8. In summary, position 7 for double bonds predominated in monoenes whereas the 7,11 isomers predominated in dienes. Both methods suggest that other positional isomers of dienes are present, although in much smaller amounts which might affect carbons 5/13, mainly 5,9 and 9,13. In the GC-MS spectrum of nonmodified heptacosadienes shown in Figure 1A, peaks 165 and 291 correspond to breakings of the bonds adjacent to double bonds. The major peaks 278 and 152 were probably obtained from a complex rearrangement, although its result is equivalent to a simple breaking of the double bonds.

*Quantitative Variations of Male Behavioral Responses as Function of Female Hydrocarbon Quantities.* Earlier work has shown that the specificity of male responses appeared rather broad: although for a dose equivalent to that borne by a single female only heptacosadiene yielded long responses, important responses could be induced by higher doses of various female unsaturates but not by female saturates. It was thus necessary to consider this problem in more

detail. To start such a study, we used the natural compounds present in female cuticle. We isolated in large amounts four diene fractions and four monoene fractions of known chain lengths (23–29 C) using a combination of thin-layer chromatographies and preparative GC. To test the specificity of male responses, each fraction was bioassayed using the procedure described above, for a range of doses close to those in vivo between 0 and 500 ng, and for higher doses to 2000 ng. The variations of the average cumulative vibration times induced in test males ($\overline{\Sigma T_N}$) with the amounts of hydrocarbons used in the bioassay ($q$) are shown in Figure 2.

A few obvious conclusions can be drawn which are valid, even if one takes into account the variability observed in measured behavioral responses. As far as dienes are concerned (Figure 2A), whatever the value of $q$, tricosadienes produce only very low responses. The three other dienes with chain lengths of 25/29 carbons yield classical dose–response curves with evidence for (1) a

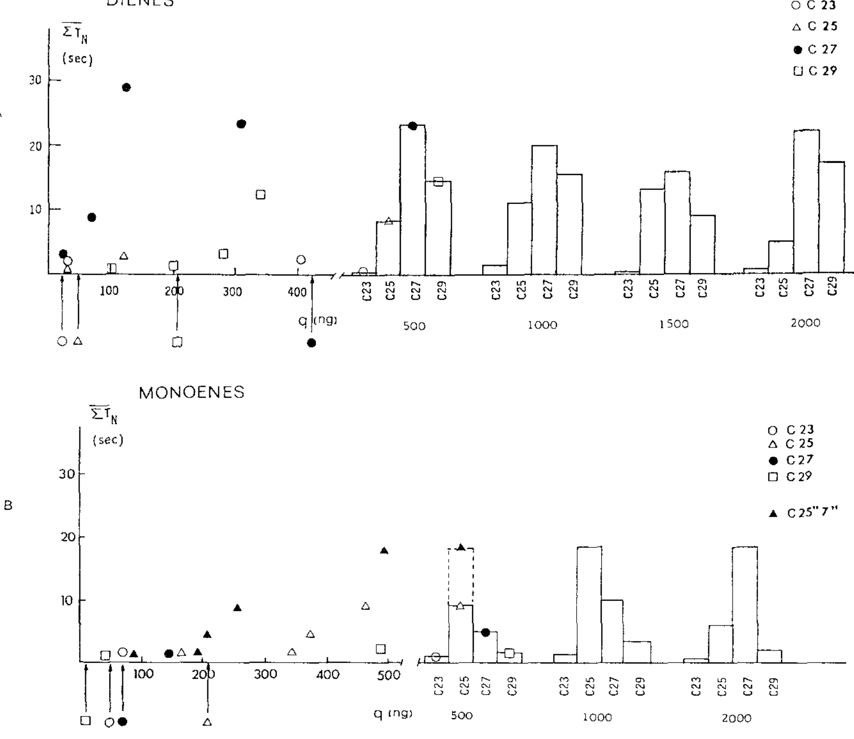


FIG. 2: Variations of induced vibration responses ($\overline{\Sigma T_N}$) of Canton S tester males with various amounts of natural hydrocarbon fractions ($q$). (A) Dienes; (B) monoenes: for pentacosenes, the curve is also represented for 7-pentacosene alone (▲). Arrows indicate doses borne by single females for each compound.

threshold, (2) an increase of $\overline{\Sigma T_N}$ values with increasing doses, and (3) a plateau which shows a saturation effect. For 100 ng of heptacosadienes, the plateau value is already reached ($\overline{\Sigma T_N} = 21 \pm 3$ sec), while values induced by comparable amounts of either pentacosadienes and nonacosadienes remain very low. It thus seems that the response threshold for heptacosadienes is much lower than those for both other dienes; for these dienes the corresponding plateau values $\overline{\Sigma T_N}$ seem also smaller: $10 \pm 4$ for pentacosadienes and $14 \pm 3$ for nonacosadienes. Figure 2B shows dose–response curves observed for monoenes; whatever the quantity, tricosenes and nonacosenes only produce low responses. Pentacosenes and heptacosenes lead to higher responses but the threshold for pentacosenes seems lower than that of heptacosenes and higher than that of heptacosadienes.

The study of the specificity of male responses shows that considering the in vivo quantities for each type of compound from the female cuticle (also shown in Figure 2A and B), only the heptacosadiene fraction is present at a level ($420 \pm 25$ ng) above the threshold and at a level for which saturation is reached. This fraction seems to correspond to the most efficient chemical signal which triggers male courtship.

The diene and monoene fractions which have a high activity consist of more or less complex mixtures of isomers (see Tables 1 and 2). For C-25 to C-29 dienes, the isomer with double bonds in positions 7 and 11 is by far the most abundant. For monoenes, isomer 7 is predominant among heptacosenes but only slightly more abundant than isomer 9 in the pentacosene fraction. As the preparative isolation of such isomers is difficult, it was easier to use synthetic isomers.

*Comparative Study of Natural and Synthetic 7,11-heptacosadienes.* Synthetic (Z,Z)-7,11-heptacosadiene was used in dose–response bioassays as previously to measure induced vibrations of tester males. Up to 100 ng, no sustained vibration was observed. Above 100 ng, the vibration increased towards a plateau ($\overline{\Sigma T_N} = 16 \pm 3$ sec) (Figure 3A). Synthetic (Z,Z)-7,11-heptacosadiene which comigrates in GC with the main natural heptacosadiene isomer is thus behaviorally active. If one compares dose–response curves of both natural and synthetic heptacosadienes consisting of a predominant molecular species with small amounts of isomers, one observes that the synthetic mixture has an efficiency slightly less than the natural one between 300 and 2000 ng, although this difference is not significant. For tested amounts above 500 ng, responses obtained with the natural (A) or synthetic (B) hydrocarbons (Figure 3) show no significant differences [$P(t) > 0.05$]. For smaller doses, the difference is more important: it looks as if the response threshold for the synthetic compound might be higher than that of the natural compound.

We also tested several synthetic monoenes: (Z)-7- and (Z)-9-tricosene, (Z)-7- and (Z)-9-pentacosene, and (Z)-9-heptacosene. No synthetic tricosene induced sustained vibrations of tester males even for a dose of 2000 ng. This is

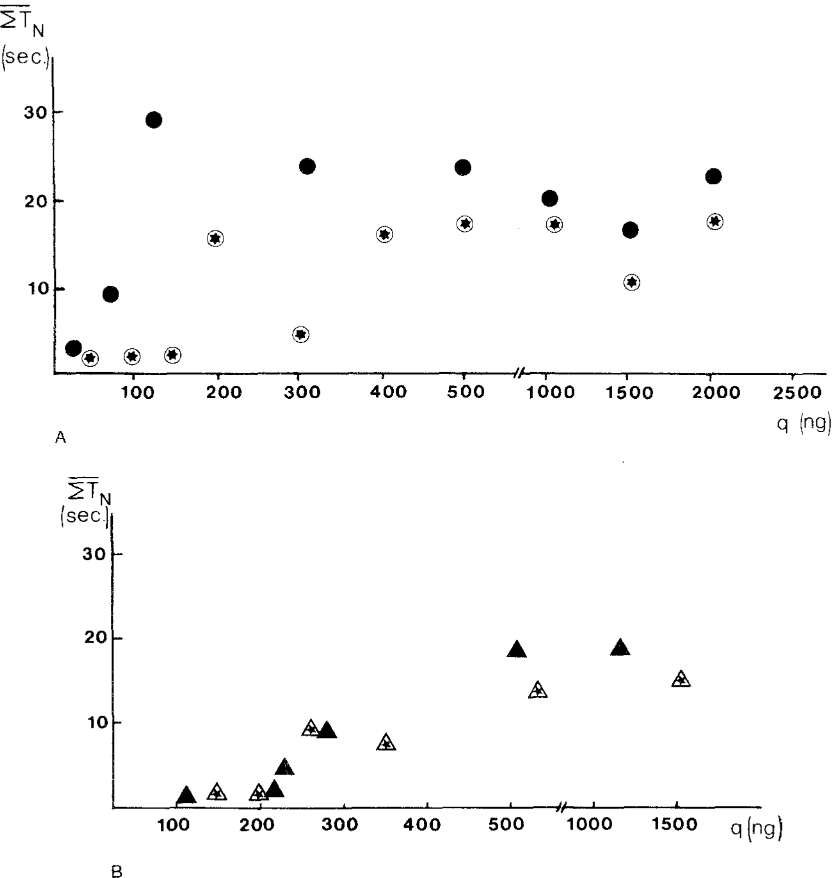


FIG. 3: Dose–response curves of the induced vibration responses ($\overline{\Sigma T_N}$) of Canton S tester males to natural and synthetic compounds as a function of the tested amount (A) heptacosadienes: ● natural 7,11-heptacosadiene; ⊛ synthetic (*Z,Z*)-7,11-heptacosadiene. (B) 7-Pentacosene: ▲ natural; ⚠ synthetic (*Z*)-7-pentacosene.

in agreement with the weak activity of the natural mixture of tricosenes. None of the (*Z*)-9-monoenes exhibited significant activity, but synthetic (*Z*)-7-pentacosene induced the dose-dependent responses shown in Figure 3B in comparison with those of the mixture of natural pentacosenes. If the latter curve is corrected for doses of 7-pentacosene present in the mixture, the obtained curve appears very similar to that corresponding to the synthetic (*Z*)-7-pentacosene.

## DISCUSSION

Cuticles of female *D. melanogaster* Canton S include several unsaturated long-chain hydrocarbons which have been shown to be potentially aphrodisiac

(Antony and Jallon, 1982). These compounds were fractionated according to their chain length and degree of unsaturation, and dose–response variations of induced wing vibrations of tester males were established for each. Only the heptacosadiene fraction was present in the cuticle at a dose higher than its estimated aphrodisiac threshold. 7,11-heptacosadiene was the main constituent, but it could not be separated from small amounts (ca. 3%) of other positional isomers. Synthetic 7,11-heptacosadienes had a behavioral activity not very different from that of the natural mixture of heptacosadienes but a small difference in estimated thresholds was observed. The synthetic product contained ca. 97.5% of (*Z,Z*)-7,11-heptacosadiene and 2.5% of two geometric isomers. It is thus clear that 7,11-heptacosadiene is a potent aphrodisiac for *D. melanogaster* Canton S males. It is suggested that 7,11-heptacosadiene with a *Z,Z* configuration is both the most active compound for males and the most abundant compound in female cuticle, but it is also possible that positional isomers also present in the natural fraction might synergize its activity. Such an hypothesis is supported by the use of a number of *D. melanogaster* mutant lines whose females have different amounts of the same 7,11-heptacosadiene isomer measured by GC. When presented to Canton S tester males, such living females induce varying levels of wing vibrations. Figure 4 shows that the intensity of male responses is positively correlated with the average dose of heptacosadiene borne by each type of female. The level of those responses are higher than those obtained in bioassays. The organization of the reconstituted hydrocarbon layer may not be identical in the bioassay situation with that of the native cuticle. Moreover, moving females are known to be more stimulatory than immobilized females (Tompkins et al., 1982) and, consequently, more than our inanimate dummies.

Other unsaturated hydrocarbons which were shown to be potential aphrodisiacs when used at high doses were present in Canton S female cuticles at doses lower than the estimated thresholds for their aphrodisiac effects. However, it is necessary to state that absolute threshold values might have been overestimated because of the bioassay itself and that aphrodisiac effects of each isolated compound might be additive or synergistic. Nevertheless, the specificity of *D. melanogaster* Canton S male responses for two structural parameters could be partially characterized.

In all behaviorally active dienes, the 7,11 isomer was predominant. Large amounts of 9-alkenes were present with the major 7 isomers in behaviorally active monoene fractions. As synthetic 9-monoenes with 23/27 carbons were not shown to induce much response, it is suggested that the more active natural monoenes have a double bond in position 7 as do the active dienes. It appears that the presence of a second double bond in position 11 enhances the biological activity of the compound.

Dienes (7,11) with 25/29 carbons and monoenes (7) with 25/27 carbons were found to have significant behavioral activity. Among dienes, heptacosadienes were optimum; among monoenes, pentacosenes were optimum. This

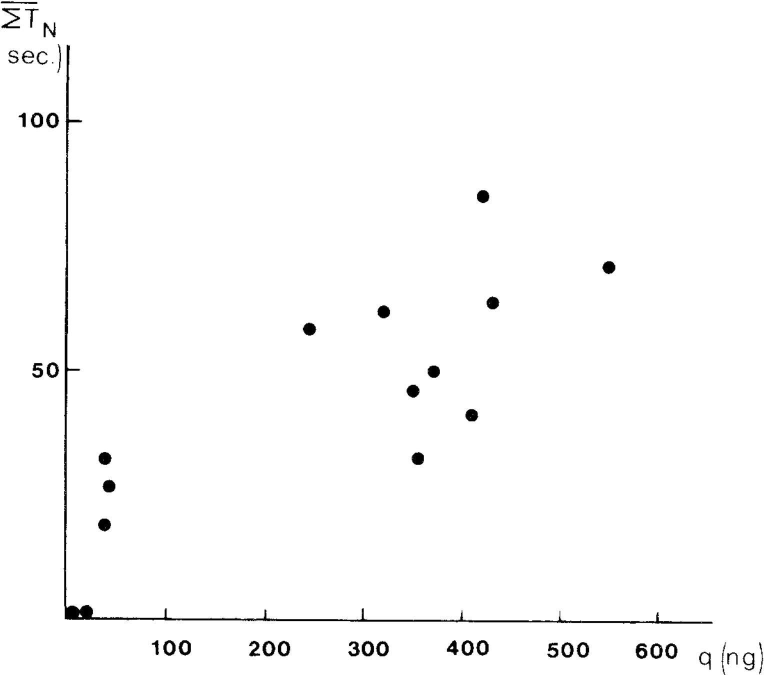


FIG. 4: Vibration responses ($\overline{\Sigma T_N}$) of tester males induced by various amounts of 7,11-heptacosadiene borne by various female individuals of different wild-type strains and mutants. The tested flies were live females from the following strains: wild types, Canton S and Tai Y. The mutant strains were either female sterile mutants: Fs(1) $ovo^{D1}$, or agametic mutant par $ecd^{1ts}$; or alleles of transformer 2: tra $2^{ts}$ bred at 16°C (tra/tra*, tra/tra$^{+}$*), bred at 18°C (tra/tra*, tra/tra**), or bred at 29°C (tra/tra**, tra/tra**); tra 2 $non^{ts}$ allele, tra/tra**, tra/tra***. * Corresponds to a female phenotype and ** to a male phenotype, but with a chromosonal female genotype.

probably corresponds to some structural requirement. Obviously there is some size constraint as tricosadiene and tricosene are inefficient. The upper size limit cannot be defined: although 29-carbon compounds are active, higher homologs were not available for testing. However, the high aphrodisiac activity of young flies of both sexes is puzzling as they have only small amounts of monoenes and dienes of 25/29 carbons, but bear complex mixtures of monoenes and dienes with 31/37 carbons (Antony, Pechine, and Jallon, unpublished results). This suggests that compounds of higher molecular weight may also be behaviorally active.

The fact that Canton S males responded to a rather broad range of unsaturated hydrocarbons suggests that the usefulness of these chemicals for sex and species recognition by flies may be limited.

*Discrimination of* D. melanogaster *Females and Males by Canton S Males.* Dienes were completely absent in Canton S males, but 7-pentacosene was present at a dose of about 100 ng/male, which is less than the threshold level and thus should not induce much courtship from conspecific males. This is exactly what has been experimentally observed (Hall, 1978; Jallon and Hotta, 1979). Thus the aphrodisiac molecules are sufficient to allow Canton S males to distinguish Canton S males from Canton S females. However, we have found males of the same species but belonging to other strains (Oregon K and Tai Y) which are much richer in 7-pentacosene: males of either strain have, respectively, ca. 380 and 560 ng of 7-pentacosene. Such males are courted by Canton S males when they are decapitated or etherized but not when they are active (Jallon, 1984). This strongly suggests that these males produce both an aphrodisiac and an inhibitory signal which is behaviorally controlled. It might be the release of the antiaphrodisiac vaccenylacetate which is produced by the ejaculatory bulb (Butterworth, 1969; Jallon et al., 1981) or a possible acoustic rejection wing display (Jallon and Ikeda, unpublished results).

*Discrimination of* D. melanogaster *Females and* D. simulans *Females by Canton S Males.* Females of all *D. melanogaster* strains which have been examined up to now contain 7,11-heptacosadiene in their cuticle, although in different amounts (Luyten, 1983). For example, Tai Y females have only 10% of the amount borne by a Canton S female; they are courted much less than Canton S females by Canton S males and mated with less. It is suggested that 7,11-heptacosadiene is a common aphrodisiac for all strains of *D. melanogaster*, although there is variability in both its production level by females and its detection characteristics by males (Jallon, 1984).

7,11-heptacosadiene and other dienes are present in only trace quantities in males and females of the sibling and sympatric species *D. simulans* (Jallon, 1984); a number of strains have been studied in this respect (Luyten, 1982). While 7-pentacosene, another potential aphrodisiac for *D. melanogaster* Canton S males, is not abundant in *D. simulans* females of most strains (e.g., Seychelles 100 ng/female), it is very abundant in a few. For example, *D. simulans* Yaounde females contain 780 ng, which is higher than the saturation level for male *D. melanogaster* Canton S. Indeed Canton S males actively court them (Jallon et al., 1982). However, neither any *D. simulans* Yaounde female nor any *D. simulans* Seychelles female copulated with a Canton S male in 30 min (Jallon, 1984). Although Canton S males displayed vigorous wing vibrations towards *D. simulans* Yaounde females, they were unable to achieve copulation: either 7-pentacosene is not an inducer for copulation, or they are rejected by the *D. simulans* females, which do not recognize aspects of their species-specific courtship song and behavior.

Such interactions are forced in the laboratory and may never take place in the field. However, they reveal that 7,11-heptacosadiene is a sex-specific and

species-specific chemical signal in all strains of *D. melanogaster*. However, chemical signals are part of a complex communication system also involving acoustic and visual signals which might be complementary and thus produce a nonambiguous sex and species recognition.

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# COMMERCIAL HOLLOW-FIBER PHEROMONE FORMULATIONS: THE DEGRADING EFFECT OF SUNLIGHT ON CELCON FIBERS CAUSING INCREASED RELEASE RATES OF THE ACTIVE INGREDIENT[1]

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**Abstract**—The effect of weathering on the release of pheromone analogs from Celcon fibers has been studied. Results from fibers exposed in Quebec indicate that UV radiation in sunlight degrades the Celcon, causing an increase in the release of the active materials. The results are discussed in the light of large interfiber release rate variation experienced with commercial fiber formulations, and the fact that the major use areas, to date, have been Arizona and California where the effect is likely to be much more pronounced. Stability of the release device to weathering is shown to be one of the many parameters to be considered in formulation design.

**Key Words**—Controlled release, hollow fibers, UV degradation, pheromones, release rates.

## INTRODUCTION

To aid in the design of economic and efficacious capillary release devices to be used for the dissemination of insect pheromones in pest monitoring and control programs, a predictive model relating the release of volatile materials to time and the length of the vapor–air column above the liquid has been developed (Weatherston et al., 1984, 1985a). The model has been shown to be valid for capillaries of various diameters and materials provided that (1) the lumen of the capillary is smooth, (2) the capillary diameter is uniform throughout its active length, and (3) there is little or no absorption of the liquid charge into the capillary wall (Weatherston et al., 1985b). These reports also indicated not only

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that pheromone-charged Celcon[3] fibers used commercially in insect control strategies performed in poor agreement with the predicted release rate but that the interfiber variation in rate was very large, most fibers releasing faster than predicted due to the rough and uneven surface of the lumen wall.

In a continuing investigation of hollow-fiber formulation design, we wished to study the effect of weathering on commercially used fibers. The ideal controlled release device should be able to withstand weathering without the efficacy being affected. From an environmental standpoint it is desirable to have a degradable device which could decompose subsequent to the field life of the pheromone formulation. In practice such a device may be difficult to realize.

During the development of Albany International's hollow-fiber controlled release system, Celcon was selected as the polymer from which the capillaries are made; one of the many reasons for this choice was that Celcon is photodegradable to formaldehyde. Although Celcon is produced in several grades, including one which is stabilized to ultraviolet radiation, the type (C-300) used to manufacture the fibers was selected because of its high flow characteristics, stability in processing, and good dimensional stability; however, it is not recommended for outdoor use because it deteriorates on continuous exposure to ultraviolet light (Anonymous, 1977). Commercial formulations are therefore possibly susceptable to a decrease in efficacy due to (1) ultraviolet-catalyzed decomposition of the pheromone compounds inside the fibers, and (2) ultraviolet-promoted decomposition of the fiber, both effects being reflected in altered pheromone release rates and a shortening of the functional longevity of the formulation.

Ultraviolet-catalyzed oxidative decomposition of diunsaturated pheromones has previously been addressed (Shani and Klug, 1980), as has the decomposition of aldehydic pheromone components in simulated field weathering studies (Shaver, 1983) and by photo-induced oxidative decomposition (Shaver and Ivie, 1982). The ultraviolet-catalyzed oxidative decomposition of aldehydic pheromones formulated in Celcon hollow fibers has been reported by Golub et al. (1983).

In this paper we wish to report the results of studies on the effect of weathering on Celcon fibers and how this affects the release rates of pheromone analogs. In addition to the factors mentioned above, which affect the validity of the predictive model, the present results indicate that the effect of weathering on the controlled release device is also critical to the design of commercial pheromone formulations.

## METHODS AND MATERIALS

The Celcon fibers were obtained from the Controlled Release Division of Albany International, Needham Heights, Massachusetts, and are of the type

[3] Celcon is the registered trademark of the Celanese Corp., Chatham, New Jersey 07928.

used commercially in their aerially broadcast insect control products. For weathering, the fibers were stapled to cards which were attached to a table, fully exposed, on the roof of the biology department at Laval University from June 23 to August 13, 1983. Exposed fibers were retrieved on days 10, 20, 30, 40, and 50, and stored in the dark until used. Hexyl acetate (99%) was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, and used without further purification. Hexadecyl acetate $^{14}C$ labelled on the carbonyl carbon atom was a gift from Dr. M. H. Benn, University of Calgary, Calgary, Alberta.

The fibers were loaded from a 10-$\mu$l syringe fitted with a fused silica needle of the type used for on-column injection in capillary gas chromatography. In the experiments involving labeled hexadecyl acetate, individual fibers were placed in miniflows as previously described (Weatherston et al., 1981), and purified dry air was pulled through the system at a rate of 1 liter/min for 21 days at 23.5°C. On days 2, 4, 7, 11, 14, 16, 18, and 21, the system was disconnected and the meniscus regression recorded. At these times the glass beads were washed with hexane (3 × 7 ml) as previously described (Weatherston et al., 1981), and the amount of hexadecyl acetate releasing from the fibers was calculated from LSC measurements. It became apparent during the experiment that breakthrough was occurring; hence the amount of hexadecyl acetate released was calculated only by meniscus regression and LSC analyses of the initial and final amounts.

Fibers loaded with hexyl acetate were placed in a fume hood at 23.7°C and a wind velocity of 2.0 m/sec, and the release was followed by meniscus regression measurements (Weatherston et al., 1985a).

## RESULTS

In order to consider a "worst case" situation, batches of empty Celcon fibers (0.203 mm ID) were weathered on the roof for 10, 20, 30, 40, and 50 days in the summer prior to being charged with active ingredient. The prevailing weather conditions at Quebec are included in Table 1. No visible changes in the fibers were observed until 20 days, when the fibers began to become chalky; subsequently they became increasingly brittle. Table 2 presents a comparison of the mean release rate, obtained from residue analyses by both scintillation counting and meniscus regression measurements of hexadecyl acetate over 21 days at 23.5°C from nonexposed fibers with that from fibers preexposed for 20 and 40 days. Although it can be seen that the mean rates are not statistically different, there is a trend for the mean release rate over 21 days to increase with the time of preexposure of the fibers. The data also reconfirm the interfiber variation in release rate and the increased rate over the predicted value (Figure 1).

Since the predicted release curve for hexadecyl acetate emitting from 0.203-mm ID capillaries at 23.5°C (Figure 1) indicates that 50% depletion of a fully charged fiber would take 7330 days an alternative faster releasing pheromone

TABLE 1. WEATHER DATA FOR JUNE THROUGH AUGUST 1983 AT QUEBEC CITY, QUEBEC (Q), AND PHOENIX, ARIZONA (P)

| | | | | | Temperature (°C) | | | |
|---|---|---|---|---|---|---|---|---|
| | Precipitation (cm) | | Sunshine (hr) | | $\overline{X}$ daily temp. | | $\overline{X}$ daily max. temp. | |
| Month | Q | P | Q | P | Q | P | Q | P |
| June (June 23–30) | 38.5 | 0 | 293.3 | 421.2 | 17.5 | 31.4 | 31.95 ± 5.80 | 39.99 ± 1.08 |
| July (July 1–31) | 73.2 | 0.96 | 241.5 | 375.5 | 19.8 | 35.3 | 33.70 ± 5.10 | 41.76 ± 2.53 |
| August (August 1–13) | 52.8 | 6.30 | 255.9 | 356.6 | 19.2 | 33.8 | 29.50 ± 3.20 | 41.00 ± 2.70 |

TABLE 2. RELEASE RATE DATA FOR $[^{14}C]$HEXADECYL ACETATE FROM 8 MIL ID CELCON FIBERS AT 23.5°C AND 21 DAYS

| Fiber | Loading (L)[a] (μg) | Residue (R) (μg) | | Amount released (L − R) (μg) | | Rate/day (μg) | |
|---|---|---|---|---|---|---|---|
| | | SC[b] | MR[c] | SC | MR | SC | MR |
| No preexposure | | | | | | | |
| 1 | 288 | 180.5 | 170.1 | 107.5 | 117.9 | 5.12 | 5.61 |
| 2 | 234 | 179.2 | 173.4 | 54.8 | 60.6 | 2.61 | 2.89 |
| 3 | 228 | 187.6 | 178.8 | 40.4 | 49.2 | 1.92 | 2.34 |
| 4 | 275 | 176.6 | 168.8 | 98.4 | 106.2 | 4.69 | 5.06 |
| 5 | 253 | 195.7 | 199.1 | 57.3 | 53.9 | 2.73 | 2.57 |
| 6 | 255 | 216.6 | 202.5 | 38.4 | 52.5 | 1.83 | 2.50 |
| $\bar{X}$ | 255.5 ± 21.1 | 189.4 ± 69.8 | 182.1 ± 13.6 | 66.1 ± 27.1 | 73.4 ± 27.8 | 3.15 ± 1.29 | 3.50 ± 1.32 |
| 20-day exposure | | | | | | | |
| 1 | 247 | 72.5 | 36.3 | 174.5 | 210.7 | 8.31 | 10.09 |
| 2 | 215 | 144.3 | 136.9 | 70.7 | 78.1 | 3.37 | 3.74 |
| 3 | 260 | 185.4 | 187.2 | 74.6 | 72.8 | 3.56 | 3.48 |
| $\bar{X}$ | 240.7 ± 18.9 | 134.1 ± 46.7 | 120.1 ± 62.7 | 106.6 ± 48.0 | 120.5 ± 63.8 | 5.08 ± 2.29 | 5.77 ± 3.06 |
| 30-day exposure | | | | | | | |
| 1 | 264 | 77.3 | 49.1 | 186.7 | 214.9 | 8.89 | 10.29 |
| 2 | 256 | 207.1 | 206.0 | 48.9 | 50.0 | 2.33 | 2.40 |
| 3 | 207 | 112.5 | 102.7 | 94.5 | 104.3 | 4.50 | 4.99 |
| $\bar{X}$ | 242.3 ± 25.2 | 132.3 ± 54.8 | 119.3 ± 65.1 | 110.0 ± 57.3 | 123.1 ± 68.6 | 5.24 ± 2.73 | 5.89 ± 3.28 |

[a] Calculated from the length of the initial liquid column.
[b] SC by scintillation counting.
[c] MR by meniscus regression.

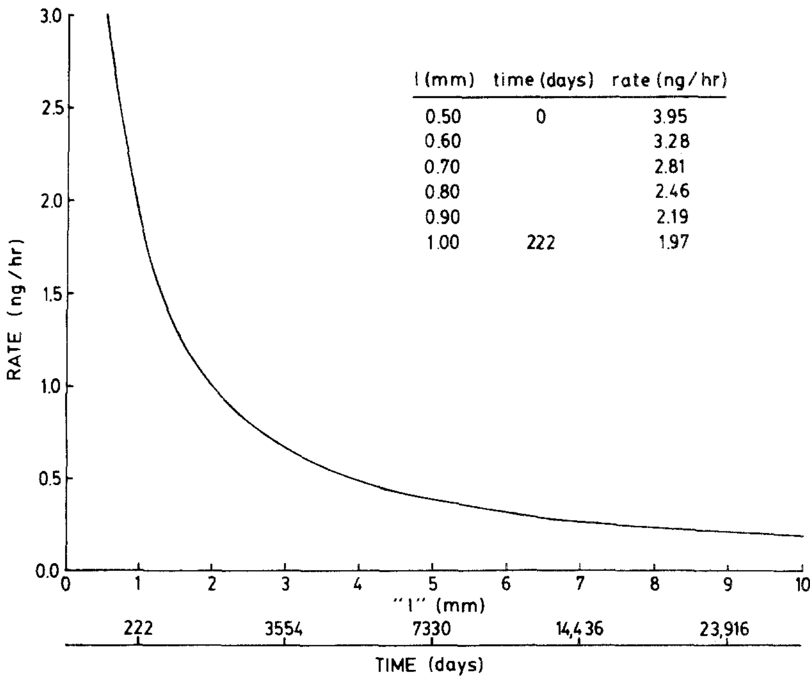



FIG. 1. Predicted release rate vs. $l$ and time, for hexadecyl acetate from 0.203-mm ID capillaries at 23.5°C.

analog was sought in order to better ascertain the reality of the trends observed with the slower releasing material. Our previous work (Weatherston et al., 1985a) indicated hexyl acetate to be a suitable compound. The curve for the predicted release of hexyl acetate with respect to the length of the vapor–air column ($l$) above the liquid and time for 0.203-mm ID capillaries at 23.7°C is shown in Figure 2. Batches of fibers ($N = 8$) which had been preexposed for 0, 10, 20, 30, and 40 days were filled with hexyl acetate, and the release rate at 23.7°C was measured by meniscus regression. Since the rate is inversely proportional to $l$ (Weatherston et al., 1984, 1985a), the data were transformed logarithmically and subjected to regression analysis for ease of presentation.

Figure 3 illustrates the data for each unexposed fiber, together with the mean rate of the eight fibers, and the predicted value. Once again, great inter-fiber variation can be seen as well as the fact that all eight fibers release at a greater rate than predicted. The mean release rates for the preexposed fibers are compared with the mean from the unexposed fibers and the predicted value in Figure 4. At $l = 1$ mm there is a clear trend that the mean release rate increases steadily from 40 $\mu$g/hr to 106 $\mu$g/hr as the preexposure is increased from 0 to 40 days. This is only valid until $l = 2.7$ mm ($\approx$ 15 hr); thereafter the mean

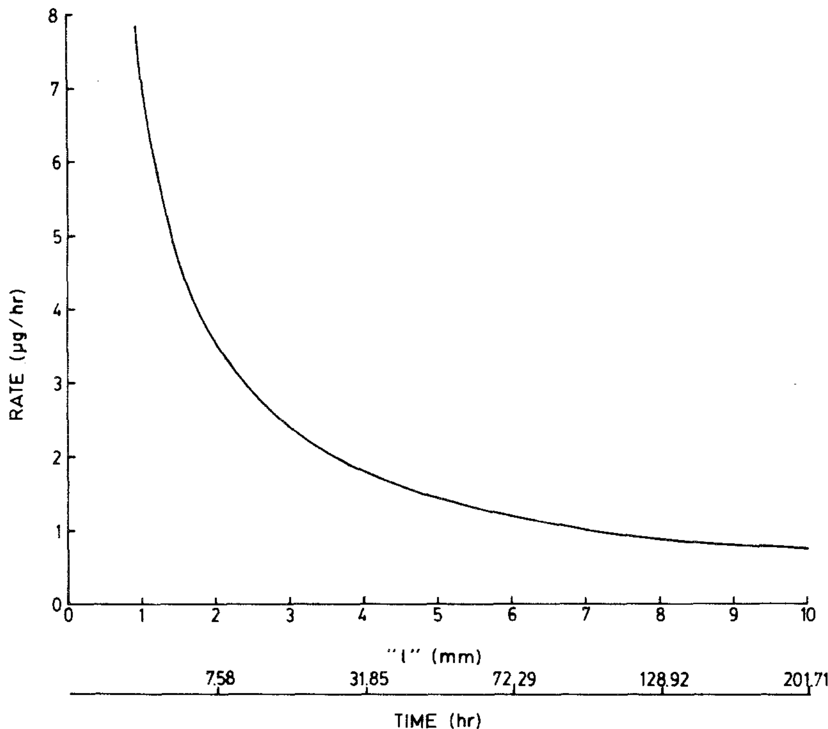



FIG. 2. Predicted release rate vs. $l$ and time, for hexyl acetate from 0.203-mm ID capillaries at 23.7°C.

release rate of the 20-day-exposed fibers is always faster than that from the unexposed and 10-day-exposed fibers; and the mean rate from the 30- and 40-day-exposed fibers is greater than that from all others. These data are alternately presented in Figure 5. Regression analysis of the means in this figure give $r$ values of 0.985, 0.942, and 0.923, respectively, at $l$ values of 1.0, 3.5, and 6.0 mm, indicating that there is a trend of increase in release rate with increased exposure.

Both the outer surface and the lumen wall of both unexposed and exposed fibers were examined by scanning electron microscopy in an attempt to observe decomposition of the fiber. Examination of the micrographs of the outer surface (Figure 6) indicates that "cracking" and "pitting" is evident in fibers exposed for 50 days but that surface decomposition, compared to unexposed fibers, is not apparent in 10- and 30-day-exposed fibers. Similarly with the lumen wall, apparent differences are only observed in fibers exposed for 50 days. The internal surface of these fibers is much more "pitted" and possesses a less "spikey" appearance than unexposed fibers (Figure 7).

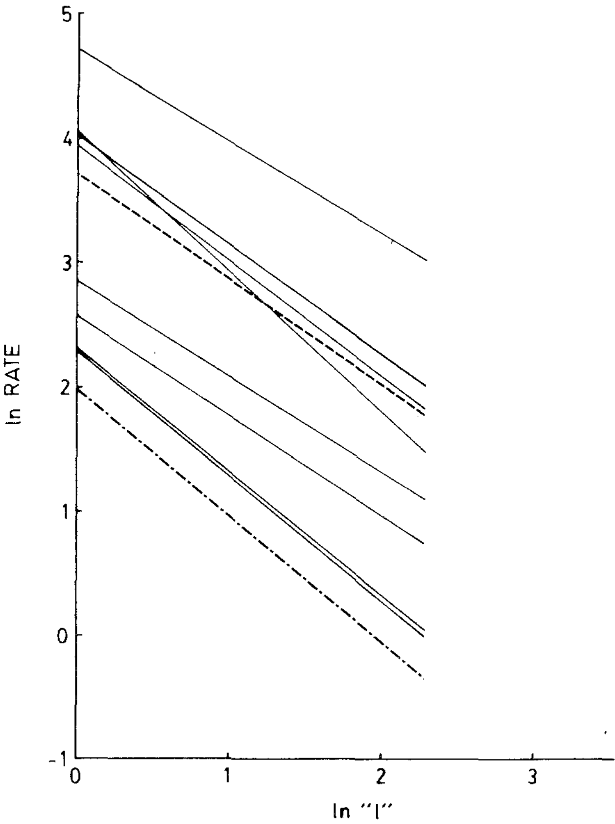



FIG. 3. Plot of ln rate vs. ln $l$ for hexyl acetate evaporated at 23.7°C from each of eight Celcon fibers (0.203 mm ID) not previously exposed to weathering (— · — · — · — · — · predicted value; ------ mean value of the eight values).

## DISCUSSION

The data presented above indicate that Celcon fibers which were preexposed to weathering release at an increasing rate with the length of exposure time. The slopes ($b$ values) obtained from the regression analysis of the means (Figure 5) are 1.63, 0.46, and 0.30 for $l$ = 1.0, 3.5, and 6.0 mm, respectively, showing that the increase is greater at $l$ = 1.0 mm than at $l$ = 3.5 mm which is greater than at $l$ = 6.0 mm. This is a reflection of both the release of volatile materials from hollow fibers (Weatherston et al., 1984, 1985a, b) and the greater likelihood of the active material being released at a site of decomposition when the length of the liquid column inside the fiber is greater. Loss of active ingredient caused by fiber degradation is corroborated by the work of Golub et al.

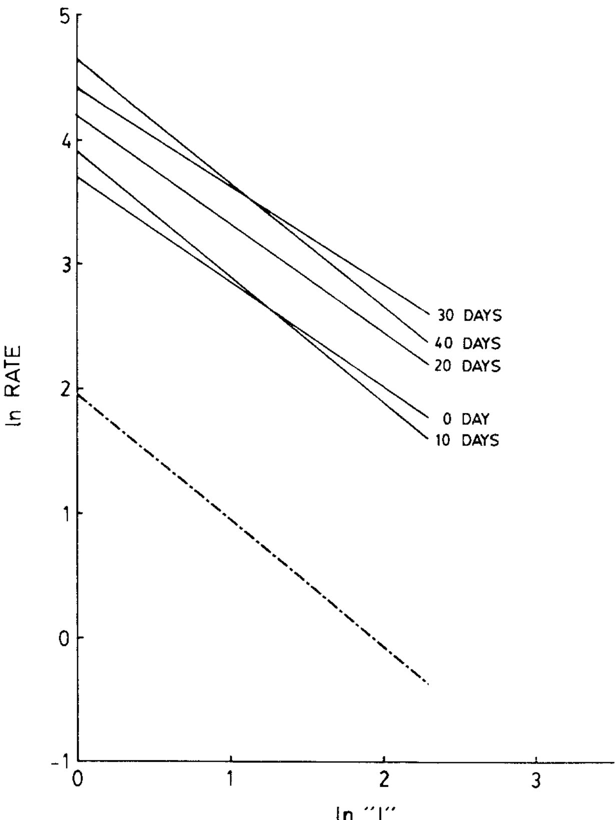



FIG. 4. Plot of ln rate vs. ln *I* for the mean release rates of hexyl acetate evaporated at 23.7°C from 0.203-mm ID Celcon fibers. The fibers ($N = 8$) were preexposed to weathering for 0, 10, 20, 30, and 40 days (— · — · — · — · predicted value).

(1983), who compared the longevity of various Celcon fiber formulations of (*E*)-11-tetradecenal exposed to weathering at Phoenix, Arizona, for 21 days. They found that after 7 days, less than 5% of the aldehyde remained in C-300 Celcon formulations containing no additives, 8% remained when the formulation included the antioxidant Banox 20BA™,[4] and 24% when the C-300 Celcon formulation included the UV stabilizer Carstab™[5] mixed with the pheromone. The use, along with the antioxidant, of black Celcon (Celcon containing carbon black) which is impervious to, and not decomposed by, UV light still contained 85% of the (*E*)-11-tetradecenal after 7 days. The authors interpreted these results to mean that the black fiber protected the aldehyde against UV-catalyzed oxidative

[4]Banox is the registered trademark of Swift & Co., Chicago, Illinois 60602.
[5]Carstab is the registered trademark of Cincinnati Milacron, Cincinnati, Ohio 45209.

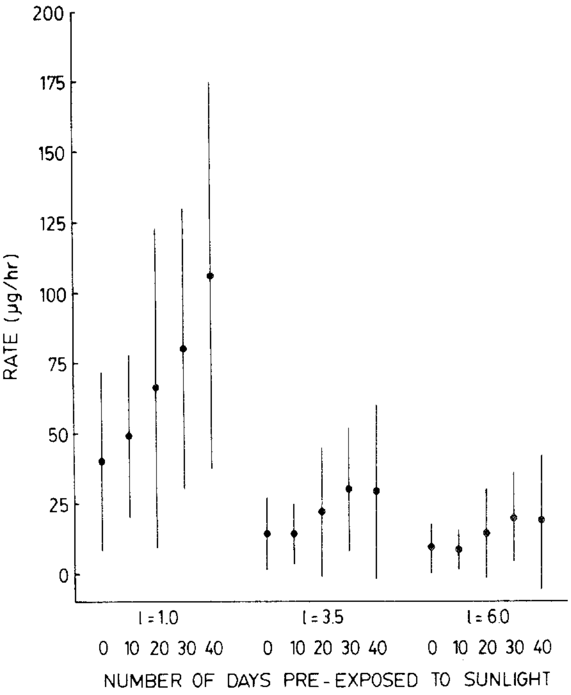
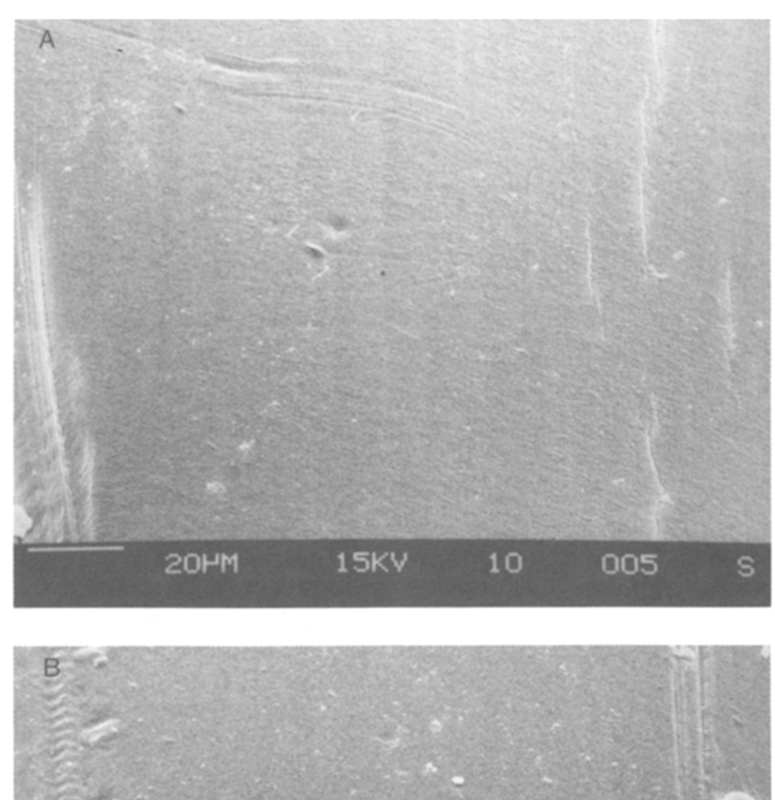



FIG. 5. Plot of mean release rate ($\mu$g/hr) for hexyl acetate from 0.203-mm ID Celcon fibers at 23.7°C, against the number of days preexposure to sunlight, at $l$ = 1.0, 3.5, and 6.0 mm.

decomposition. While this is indeed correct, the UV stabilizer Carstab afforded some protection (from < 5% to 24%) and hence the increased longevity of the black fiber formulation is the sum of the effects caused by the antioxidant (minor) and the protection given to both the aldehyde and the Celcon.

The pre-exposure of the fibers in the present study was carried out at Quebec City. As can be seen from Table 1, had they been exposed at Phoenix, they would have been subjected to longer periods of sunshine and more intense UV radiation, which would accelerate the degradation of the Celcon. Celcon fibers (C-300) exposed for various durations at Phoenix in the summer of 1980 became chalky, then brittle with increasing exposure, and had completely fragmented in 60 days (Weatherston and Phelan, unpublished).

The major commercial use of hollow Celcon fiber formulations of pheromones has been in the control of pink bollworm in Arizona and California (> 6000 kg of formulation on 125,000 acres in 1981); hence the effect of weathering on the release rate found in the present study would be less than that occurring under field use situations.



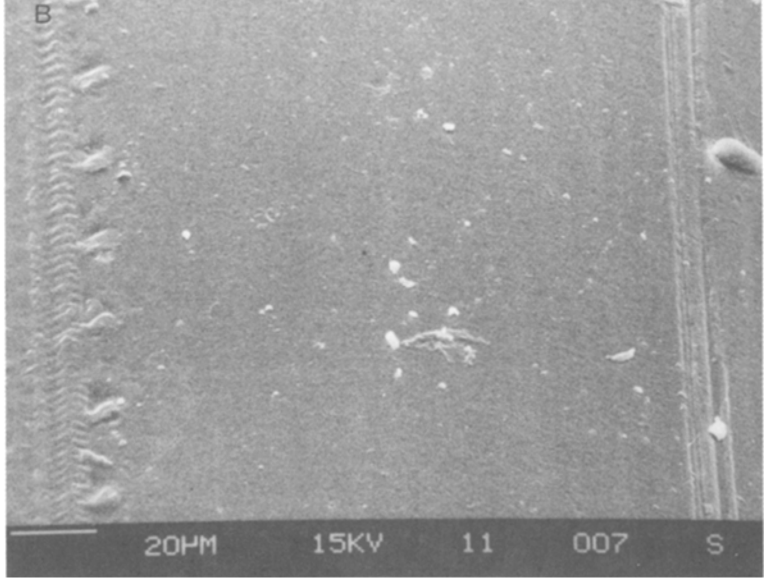
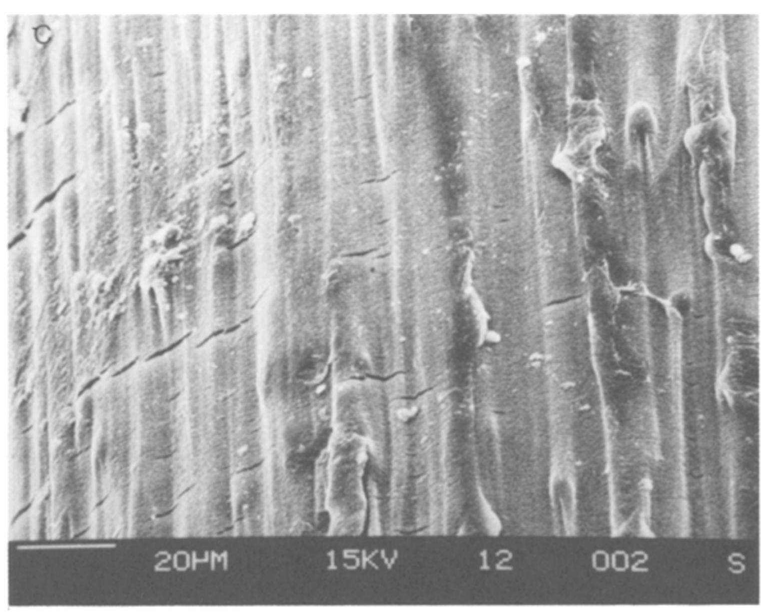




FIG. 6. Scanning electron micrographs of the outer surface of Celcon fibers preexposed to weathering for various time periods: (A) 0 days, (B) 10 days, (C) 30 days, and (D) 50 days.



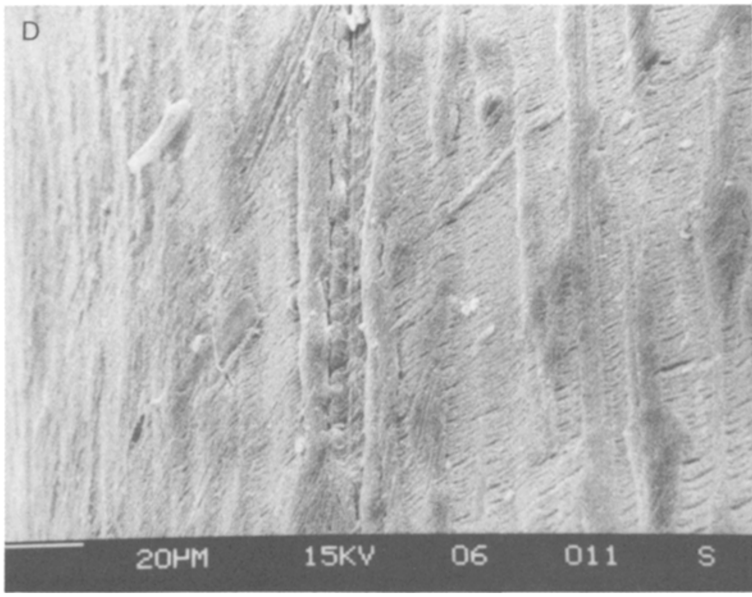




FIG. 6. Continued.

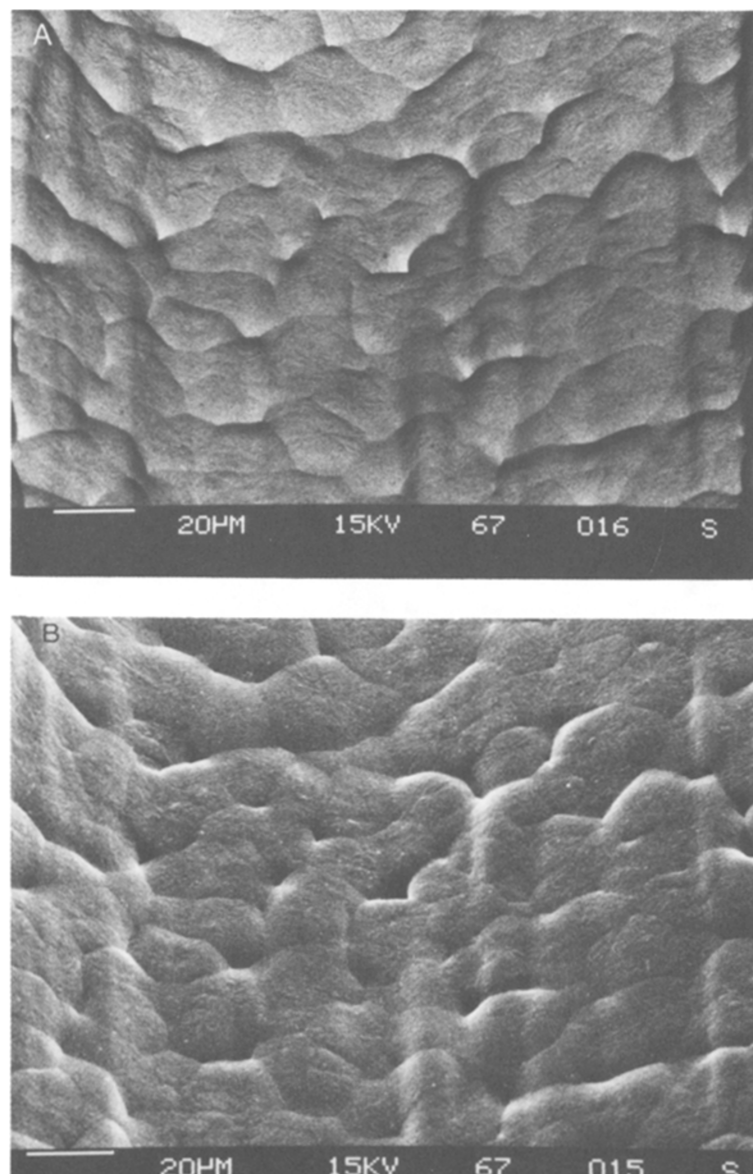



FIG. 7. Scanning electron micrographs of the lumen wall of Celcon fibers: (A) unexposed, and (B) preexposed for 50 days.

In the design of hollow fiber formulations of pheromones, factors influencing the efficacy and economics include the choice of fiber material such that it may be extruded to yield fibers whose lumen walls are smooth, the internal capillary diameter is uniform throughout its active length, and there is little or no absorption of the pheromone into the fiber wall (Weatherston et al, 1985b). The results reported here emphasize that the ability to withstand weathering is also an important parameter which should be considered in the choice of fiber material.

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# ATTRACTION OF MOTH SPECIES OF TORTRICIDAE, GELECHIIDAE, GEOMETRIDAE, DREPANIDAE, PYRALIDAE, AND GRACILLARIIDAE FAMILIES TO FIELD TRAPS BAITED WITH CONJUGATED DIENES

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**Abstract**—Field surveys of a series of conjugated diunsaturated straight-chain primary alcohols, acetates, and aldehydes (dienes), including the 3,5-dodecadienyl, 8,10-dodecadienyl, 3,5-tetradecadienyl, 8,10-tetradecadienyl, 9,11-tetradecadienyl, and 10,12-tetradecadienyl primary alcohols, acetates, and most aldehydes, and the related monounsaturated straight-chain primary alcohols, acetates, and aldehydes (monoenes), including the 3-dodecadienyl alcohol and acetates, attracted the following species from the title families. Diene attractants have been demonstrated for at least one *Phyllonorycter* sp., *Chionodes lugubrella* (Fabr.), *Leptostales ferruminaria* (Zell.), *Drepana bilineata* (Pack.), *Pyrausta fodinalis* (Led.), *Notocelia purpurissatana* Heinr., *Phaneta alterana* Heinr. Monoene attractants were demonstrated for *Hedya separatana* (Kft.), *Cydia fletcherana* (Kft.), *Barbara mappana* Free., *Ancylis burgessiana* (Zell.), *Ancylis nubeculana* Clem., *Evippe prunifoliella* Cham., *Phlyctaenia coronata tertialis* (Gn.), a *Chionodes* sp., a Gelechiidae sp., and an unidentified *Phyllonorycter* sp. Replicated field experiments showed attraction-inhibition relationships for most species. Electroantennagram data are presented for *Leptostales ferruminaria* and *Drepana bilineata*.

**Key Words**—Sex attractant, Lepidoptera, Tortricidae, Gelechiidae, Geometridae, Drepanidae, Pyralidae, Gracillariidae, conjugated diene.

## INTRODUCTION

Field surveys of the geometrical isomers of straight-chain conjugated diunsaturated 12-carbon primary alcohols, acetates, and aldehydes (dodecadienes) have been successful in providing synthetic sex attractants for a large number of Lep-



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idoptera. The 8,10-dodecadienes and 7,9-dodecadienes have been reported as attractants or pheromone components for 27 species of Tortricidae (Reed and Chisholm, 1985; Chisholm et al., 1985; Roelofs and Brown, 1982). The 9,11-dodecadienes have been reported as attractants or pheromone components for four Tortricidae species (Chisholm and Reed, 1985, Hill et al., 1981, Bjostad et al., 1980), one Noctuidae (Nesbitt et al., 1975), one Cosmopterigidae and one Limacodidae (Chisholm and Reed, 1985). The 5,7-dodecadienes have been reported as attractants or pheromone components for one Tortricidae species (Chisholm et al., 1985), five Lasiocampidae species (Chisholm et al., 1980a, Underhill et al., 1980, Vu et al., 1980, Meng, 1983, Priesner et al., 1984), and three Noctuidae species (Reed et al., 1984).

This report presents data from field surveys of conjugated dienes and monoenes alone and in various combinations. Additional field tests using the 8,10-dodecadienes and their monoene analogs have produced improved lures for two species and new sex attractants for four others. Most of the dodecadienes have been surveyed, and the majority of attracted species were from the subfamily Olethreutinae. Since the majority of reported attractants for Olethreutinae are dodecenyl compounds, the 3,5-dodecadienes were surveyed to complete the odd-numbered dodecadiene series. Since most of the reported attractants for Tortricinae subfamily are monounsaturated straight-chain 14-carbon primary alcohols, acetates, and aldehydes (tetradecenes), it is possible that the diunsaturated straight-chain 14-carbon primary alcohols, acetates, and aldehydes (tetradecadienes) may provide attractants for that subfamily. The 3,5-tetradecadienes, 8,10-tetradecadienes, 9,11-tetradecadienes, and 10,12-tetradecadienes were also prepared and field screened for possible attractancy. We also tested for synergism and inhibition interactions of conjugated dienes and monoenes.

## METHODS AND MATERIALS

The chemicals used in this study were synthesized and purified in this laboratory. The synthesis of the 8,10-dodecadien-1-ols, 8-dodecen-1-ols, and the 10-dodecen-1-ols were previously described by Chisholm et al., (1985), and the 3,5-tetradecadien-1-ols, 8,10-tetradecadien-1-ols, 9,11-tetradecadien-1-ols, 10,12-tetradecadien-1-ols, 3,5-dodecadien-1-ols, tetradecen-1-ols, and dodecen-1-ols were prepared by similar methods, except (*Z*,*Z*)-9,11-tetradecadien-1-ol (*Z*9,*Z*11–14:OH) which was prepared by Wittig condensation (Reed and Chisholm, 1985). All the alcohols were purified to greater than 99% isomeric purity by argentation chromatography (Houx et al., 1974). The acetates were prepared by treatment of the appropriate alcohol with hot acetic anhydride (Reed and Chisholm, 1985), and the aldehydes were prepared by oxidation of the appropriate alcohol with pyridinium chlorochromate (Corey and Suggs, 1975). 3,5-Dodecadienals and 3,5-tetradecadienals were not stable when prepared by this

method so they were not included in the survey. Structures were verified by NMR, IR spectroscopy and mass spectroscopy. Isomeric purity was checked on a Hewlett Packard gas chromatograph model 5710 with flame ionization detector using a DB-5 fused silica capillary column (0.32 mm ID × 30 m; J & W Scientific Inc Rancho Cordova, California). All compounds used were greater than 98% pure.

The chemicals in hexane solution were impregnated into rubber septa (Arthur H. Thomas Co, Phila., Pennsylvania No. 8753-D22) and then protected from oxidation with 0.1 ml of 10% butylated hydroxytoluene (BHT) in acetone. Single-component lures received a 100-μg dose of chemical; in two-component lures the dose was 110 μg with the minor component at 10 μg unless otherwise stated. The Pherocon 1-CP traps (Zoecon Corp., Palo Alto, California) containing the septa were hung on tree branches or fence lines, 1–2 m above the ground and spaced 15 m apart. Trap captures were recorded twice weekly, sticky liners were replaced as required, and the lures were renewed every four weeks.

Dienes were initially surveyed as single-component lures except the 10,12-tetradecadienes which contained two-component lure mixtures as well as single components. The second components were chosen on the basis of a difference in either the functional group or the geometry of the double bonds from the attracting compound. In cases where captures from the survey initiated replicated experiments, the second components were chosen as previously mentioned and the analogous monoenes were tested as second components and single components.

Field trapping was carried out 100 km northeast of Saskatoon, Saskatchewan, Canada, in a forest containing white spruce, Jack pine, birch, aspen, and numerous herbaceous shrub species. Field trapping was also carried out near Saskatoon in an area bordering on cultivated land, a tree nursery, and native prairie. Electroantennogram (EAG) response of the insects antennae to synthetic chemicals were recorded (Chisholm et al., 1975) using field-trapped males.

Data from field tests that were replicated three times and set out in randomized block design, were transformed $\sqrt{X + 0.5}$ where $X$ is the number of moths captured per trap. They were then submitted to analysis of variance; significantly different means were separated by Duncan's multiple-range test.

## RESULTS AND DISCUSSION

Table I summarizes the results of the overall survey. Table 2 presents the data from a field experiment using (*E*,*E*)-8,10-dodecadienyl acetate (*E*8,*E*10–12:Ac), (*E*,*Z*)-8,10-dodecadienyl acetate (*E*8,*Z*10–12:Ac), and the analogous dodecenyl acetates alone or in various combinations. The data show that *Grapholita lunatana* (Walsingham) is attracted to *E*8,*E*10–12:Ac, *Cydia toreuta* (Grote) is attracted to *E*8,*Z*10–12:Ac, and *Cydia ingrata* (Heinrich) is attracted to a combination of *E*8,*E*10–12:Ac + *E*8,*Z*10–12:Ac in 1:1 ratio. All three

TABLE 1. LEPIDOPTERA SPECIES ATTRACTED TO TRAPS DURING SURVEYS IN 1984

| Species | Attractant | Dose (μg) | No. males trapped | Trapping period |
|---|---|---|---|---|
| Tortricidae: Olethreutinae | | | | |
| Tribe Laspeyresiini | | | | |
| *Grapholita lunatana*[a] | *E*8,*E*10–12 : Ac | 100 | 5759 | May 7–June 15 |
| *Grapholita* sp.[a] | *E*8–12 : Ac | 100 | 254 | May 4–June 18 |
| *Grapholita prunivora*[b] | *Z*8–12 : Ac | 100 | 461 | June 4–July 16 |
| *Cydia toreuta*[a] | *E*8,*Z*10–12 : Ac | 100 | 629 | June 4–June 21 |
| *Cydia ingrata*[a] | *E*8,*E*10–12 : Ac+*E*8,*Z*10–12 : Ac | 100 : 100 | 1518 | May 14–June 12 |
| *Cydia fletcherana* | *E*8–12 : Ac | 100 | 44 | June 29–July 9 |
| Tribe Olethreutini | | | | |
| *Hedya separatana* | *Z*10–12 : Ac | 100 | 175 | May 14–July 12 |
| Tribe Eucosmini | | | | |
| *Phaneta alterana* | *E*8,*E*10–12 : Ac+*E*10–12 : Ac | 100 : 10 | 16 | July 9–July 16 |
| *Barbara mappana* | *E*9–12 : Ac | 100 | 9 | May 14–June 4 |
| *Ancylis burgessiana* | *E*9–12 : Ac | 100 | 101 | June 12–July 9 |
| *Ancylis nubeculana* | *Z*9–12 : Ac | 100 | 13 | June 18–June 28 |
| *Notocelia purpurissatana* | *Z*10,*Z*12–14 : Ac, or, | 10 | 122 | June 28–July 30 |
| | *Z*10,*Z*12–14 : OH | 100 | | |
| Gelechiidae: Gelechiinae | | | | |
| Gelechiidae sp. | *Z*5–12 : Ac | 100 | 190 | Sept. 8–Sept. 30[c] |
| *Chionodes* sp. | *E*3–12 : Ac | 100 | 133 | July 4–Aug. 13 |

| | | | | |
|---|---|---|---|---|
| *Evippe prunifoliella* | *Z*3–12 : Ac | 100 | 56 | June 25–July 12 |
| *Chionodes lugabrella* | *E*3,*Z*5–12 : Ac+*Z*3,*E*5–12 : Ac | 100 : 10 | 1812 | June 12–July 12 |
| Geometridae | | | | |
| *Leptostales ferruminaria* | *Z*9,*Z*11–14 : Ald+*Z*9,*Z*11–14 : OH | 100 : 10 | 32 | May 29–June 26 |
| Drepanidae | | | | |
| *Drepana bilineata* | *Z*9,*E*11–14 : Ald | 100 | 12 | May 25, July 7–21[d] |
| Pyralidae: Pyraustinae | | | | |
| *Pyrausta fodinalis* | *E*10,*E*12–14 : Ac | 100 | 131 | July 12–Aug. 20 |
| *Phlyctaenia coronata tertialis* | *Z*9–12 : Ac | 100 | 12 | June 21–July 4 |
| Gracillariidae | | | | |
| *Phyllonorycter* sp. (1) | *E*10–12 : Ac | 100 | 990 | May 14–July 16 |
| *Phyllonorycter* sp. (2) | *E*8,*E*10–14 : Ac | 100 | 489 | May 28–June 8, July 12–Aug. 13 |
| | *Z*8,*Z*10–14 : Ald | 100 | 1090 | May 28–June 18 |

[a] Previously reported (Chisholm et al., 1985).
[b] Previously reported (Roelofs and Cardé, 1974).
[c] 1982 trapping period.
[d] 1983 trapping period.

TABLE 2. ATTRACTION OF SEVERAL OLETHREUTINAE SPECIES TO 8,10-DODECADIENYL ACETATES AND RELATED DODECENYL ACETATES, MAY 4–JULY 16, 1984

| Lure composition (μg) | Total males captured[a] | | | |
|---|---|---|---|---|
| | *G. lunatana* | *C. ingrata* | *Grapholita* sp. | *C. toreuta* |
| *E*8,*E*10–12 : Ac(100) | 917 ab | 10 h | 0 | 0 k |
| *E*8,*Z*10–12 : Ac(100) | 0 e | 5 h | 0 | 134 i |
| *Z*8–12 : Ac(100) | 0 e | 0 h | 0 | 0 k |
| *E*8–12 : Ac(100) | 1 e | 0 h | 254 | 0 k |
| *Z*10–12 : Ac(100) | 0 e | 0 h | 0 | 0 k |
| *E*10–12 : Ac(100) | 3 de | 0 h | 0 | 0 k |
| *E*8,*E*10–12 : Ac(100) + *E*8,*Z*10–12 : Ac(10) | 437 c | 4 h | 0 | 0 k |
| *E*8,*E*10–12 : Ac(100) + *E*8,*Z*10–12 : Ac(100) | 8 de | 379 f | 0 | 103 ij |
| *E*8,*E*10–12 : Ac(10) + *E*8,*Z*10–12 : Ac(100) | 0 e | 7 h | 0 | 147 i |
| *E*8,*E*10–12 : Ac(100) + *E*8,*Z*10–12 : Ac(100) + *E*8–12 : Ac(10) | 7 de | 275 fg | 4 | 96 ij |
| *E*8,*E*10–12 : Ac(100) + *E*8,*Z*10–12 : Ac(100) + *Z*8–12 : Ac(10) | 3 de | 345 fg | 0 | 28 ijk |
| *E*8,*E*10–12 : Ac(100) + *E*8,*Z*10–12 : Ac(100) + *E*10–12 : Ac(10) | 36 de | 338 fg | 0 | 134 i |
| *E*8,*E*10–12 : Ac(100) + *E*8,*Z*10–12 : Ac(100) + *Z*10–12 : Ac(10) | 5 de | 149 g | 0 | 11 jk |
| *E*8,*E*10–12 : Ac(100) + *E*8–12 : Ac(10) | 703 bc | 1 h | 0 | 0 k |
| *E*8,*E*10–12 : Ac(100) + *Z*8–12 : Ac(10) | 109 d | 1 h | 0 | 1 k |
| *E*8,*E*10–12 : Ac(100) + *E*10–12 : Ac(10) | 1168 a | 12 h | 0 | 0 k |
| *E*8,*E*10–12 : Ac(100) + *Z*10–12 : Ac(10) | 488 c | 24 h | 0 | 0 k |

[a] Numbers within column followed by the same letter are not different ($P = 0.05$).

species have overlapping flight periods (Table 1) and little is known about their mating habits, so species isolation may be maintained by chemical means. Chisholm et al. (1985) have previously shown that the capture of *G. lunatana* by *E*8,*E*10–12 : Ac was slightly reduced by *E*8,*Z*10–12 : Ac in 10 : 1 ratio, and this combination then attracted *C. ingrata*. They also showed that addition of (*Z*)-8-dodecenyl acetate (*Z*8–12 : Ac) inhibited *G. lunatana* capture. Table 2 shows that *E*8,*E*10–12 : Ac + *E*8,*Z*10–12 : Ac in 10 : 1 ratio is not an effective lure for *C. ingrata* when placed in direct competition with the same components at a 1 : 1 ratio. In addition to *C. ingrata*, *C. toreuta* was captured by the lure at 1 : 1 ratio. The addition of *Z*8–12 : Ac reduced *C. toreuta* captures from 103 to 28, while addition of *Z*10–12 : Ac reduced its captures from 103 to 11. Increasing the ratio of either monoene in the bait may improve the specificity of the lure for *C. ingrata*. In addition to the species reported in Table 2, all four monoene acetates as single components at a 100-$\mu$g dose attracted other lepidoptera: *Z*10–12 : Ac attracted *Hedya separatana* (Kearfott); (*E*)-10-dodecenyl acetate (*E*10–12 : Ac) attracted *Phyllonorycter* sp. (1); *Z*8–12 : Ac attracted *Grapholita prunivora* (Walsh); and (*E*)-8-dodecenyl acetate (*E*8–12 : Ac) attracted males of an undescribed *Grapholita* sp. and *Cydia fletcherana* (Kearfott).

In a competitive trapping situation when any of these monoenes were in combination with *E*8,*E*10–12 : Ac or *E*8,*Z*10–12 : Ac, no *H. separatana*, *C. fletcherana*, *Phyllonorycter* sp. (1), or *G. prunivora* were captured and the *Grapholita* sp. captures were reduced significantly (Table 2). Chisholm et al. (1985) had previously shown both *Grapholita* sp. and *G. prunivora* captures were inhibited by *E*8,*E*10–12 : Ac. The combination of *E*8,*E*10–12 : Ac + *E*10–12 : Ac (10 : 1) did, however, attract *Phaneta alterana* Heinrich when neither single component was effective (Table 1).

Table 1 shows that both the *Grapholita* sp. and *C. fletcherana* were attracted to *E*8–12 : Ac, but these species are isolated by their flight periods. No sex attractant has previously been reported for *H. separatana*, but Voerman (1979) has reported that *Z*10–12 : Ac attracts *H. atropunctana* (Zetterstedt), and Arn et al. (1974) have reported *Z*10–12 : Ac is an attractant inhibitor of *H. nubiferana* (Haworth). *Phyllonorycter* specimens captured by *E*10–12 : Ac were not identified to species, but Roelofs et al. (1977) have reported that *P. blancardella* (F.) is attracted to *E*10–12 : Ac. The 9-dodecenyl acetates were surveyed concurrently with the dienes, and (*E*)-9-dodecenyl acetate (*E*9–12 : Ac) attracted *Barbara mappana* Freeman and *Ancylis burgessiana* (Zeller), while *(Z)-9-dodecenyl acetate* (*Z*9–12 : Ac) attracted *Ancylis nubeculana* (Clemens) and *Phlyctaenia coronata tertialis* (Guenee). Attractants have not previously been reported for these four species.

Field survey of the single-component 3,5-tetradecadienes attracted two species. *Acossus centerensis* (Lintner) was attracted to (*E*,*E*)-3,5-tetradecadienyl acetate, and *Prionoxystus robiniae* (Packard) was attracted to (*Z*,*E*)-3,5-tetradecadienyl acetate. Both have been reported previously by Doolittle et al. (1976).

The single component 3,5-dodecadiene survey captured *Chionodes lugubrella* (F.). A subsequent replicated test (Table 3) suggests the best lure is (*E*,*Z*)-3,5-dodecadienyl acetate + (*Z*,*E*)-3,5-dodecadienyl acetate in 10 : 1 ratio. Addition of analogous monoenes to the bait did not significantly alter moth captures, but Table 1 shows (Z)-3-dodecenyl acetate (*Z*3–12 : Ac) as a single component at 100 μg caught *Evippe prunifoliella* Chambers and (*E*)-3-dodecenyl acetate (*E*3–12 : Ac) at 100 μg caught a *Chionodes* sp., while neither moth was caught when these monoenes were combined with a diene. The 3-dodecenyl acetates as well as the 3,5-dodecadienyl acetates have not previously been reported as sex attractants. All three species captured are from the Gelechiidae family.

Another gelechiid was attracted to (Z)-5-dodecenyl acetate (*Z*5–12 : Ac) (Table 4); however, when (Z)-7-dodecenyl acetate (*Z*7–12 : Ac) was added to the lure at the 1% level, the Gelechiidae sp. was excluded and *Euxoa ochrogaster* (Guenee) was captured. The *Z*5–12 : Ac + *Z*7–12 : Ac has been reported attractive to *E. ochrogaster* (Steck et al., 1980). The table also shows that (Z,Z)-5,7-dodecadienyl acetate (*Z*5,*Z*7–12 : Ac) and (*Z*,*E*)-5,7-dodecadienyl acetate (*Z*5,*E*7–12 : Ac) will substitute for the *Z*7–12 : Ac in the lure, inhibiting the capture of the Gelechiidae sp. and acting as a coattractant for *E. ochrogaster*. It

TABLE 3. CAPTURES OF *Chionodes lugubrella* IN TRAPS BAITED WITH 3,5-DODECADIENES, JUNE 21–JULY 14, 1983, AND JUNE 25–JULY 16, 1984

| | Total males captured[a] | |
|---|---|---|
| Lure composition (μg) | 1983 | 1984 |
| *E*3,*Z*5-12 : Ac(100) | 76 f | 112 bcd |
| *E*3,*Z*5-12 : Ac(100) + *E*3,*Z*5-12 : OH(10) | 37 f | 35 d |
| *E*3,*Z*5-12 : Ac(100) + *E*3,*E*5-12 : Ac(10) | 72 f | 86 cd |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(10) | 219 e | 232 abc |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*Z*5-12 : Ac(10) | 117 ef | 50 d |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(1) | — | 96 bcd |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(100) | — | 215 abc |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(10) + *Z*3-12 : Ac(10) | — | 155 abcd |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(10) + *E*3-12 : Ac(10) | — | 279 ab |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(10) + *Z*5-12 : Ac(10) | — | 310 a |
| *E*3,*Z*5-12 : Ac(100) + *Z*3,*E*5-12 : Ac(10) + *E*5-12 : Ac(10) | — | 231 ab |
| *Z*3-12 : Ac(100) | — | 0 |
| *E*3-12 : Ac(100) | — | 0 |
| *Z*5-12 : Ac(100) | — | 0 |
| *E*5-12 : Ac(100) | — | 0 |

[a] Numbers within column followed by the same letter are not different ($P = 0.05$). (−) indicates these mixtures were not tested in 1983.

TABLE 4. ATTRACTION AND INHIBITION OF A GELECHIIDAE SPECIES, SEPTEMBER 14–OCTOBER 15, 1982

| Lure composition (μg) | Total males captured[a] | |
|---|---|---|
| | Gelechiidae sp. | *Euxoa ochrogaster* |
| Z5-12:Ac(100) | 117 a | 3 e |
| Z5-12:Ac(100) + Z7-12:Ac(1) | 0 b | 36 d |
| Z5-12:Ac(100) + Z5,Z7-12:Ac(1) | 73 a | 0 e |
| Z5-12:Ac(100) + Z5,Z7-12:Ac(10) | 0 b | 0 e |
| Z5-12:Ac(100) + Z5,Z7-12:Ac(100) | 0 b | 45 d |
| Z5-12:Ac(100) + Z5,Z7-12:Ac(1000) | 0 b | 73 c |
| Z5-12:Ac(100) + Z5,*E*7-12:Ac(100) | 0 b | 28 d |
| Z5-12:Ac(100) + *E*5,Z7-12:Ac(100) | 0 b | 2 e |

[a] Numbers within column followed by the same letter are not different ($P = 0.05$).

should be noted that no Z7–12:Ac was detected in the 5Z,7Z–12:Ac or the Z5,*E*7–12:Ac (<0.01%). Several other gelechiid have been reported attracted to Z5–12:Ac or its *E* isomer (Roelofs and Comeau, 1971; Ando et al., 1975, 1977).

The two-component 10,12-tetradecadiene survey caught two species. *Pyrausta fodinalis* (Lederer) were captured by traps containing (*E,E*)-10,12-tetradecadienyl acetate at 100 μg and were excluded only when the second component was (*E,E*)-10,12-tetradecadien-1-ol. *Notocelia purpurissatana* Heinrich were caught in traps baited with either (*Z,Z*)-10,12-tetradecadienyl acetate at 10-μg dose or (*Z,Z*)-10,12-tetradecadien-1-ol at 100-μg dose. Further experiments may produce a more definitive lure for these two species. Recently, McDonough et al. (1982) isolated (*E,E*)-10,12-tetradecadienyl acetate and (*E,Z*)-10,12-tetradecadienyl acetate from *Amorbia cuneana* (Walsingham) and have shown they are pheromone components.

The survey using single component 9,11-tetradecadienes trapped two lepidopteran species (Table 1). A small number of *Drepana bilineata* (Packard) were captured by (*Z,E*)-9,11-tetradecadienal (Z9,*E*11–14:Ald) at 100-μg dose. *Leptostales ferruminaria* (Packard) was also caught in small numbers over several years by traps containing (*Z,Z*)-9,11-tetradecadienal (Z9,Z11–14:Ald). In 1984, 25 of 32 *L. ferruminaria* captured were attracted to Z9,Z11–14:Ald + (*Z,Z*)-9,11-tetradecadien-1-ol (Z9,Z11–14:OH) in 10:1 ratio. Low capture numbers may be due to incomplete baits or to low populations, but EAG data for each of the species shows substantial activity for its field attractant (Figures 1 and 2). *L. ferruminaria* shows a relatively large response for Z9,Z11–14:Ald (Figure 1) as well as its *Z,E* and *E,Z* isomers, but the analogous monoenes showed no significant EAG activity. *D. bilineata* (Figure 2) gave the largest

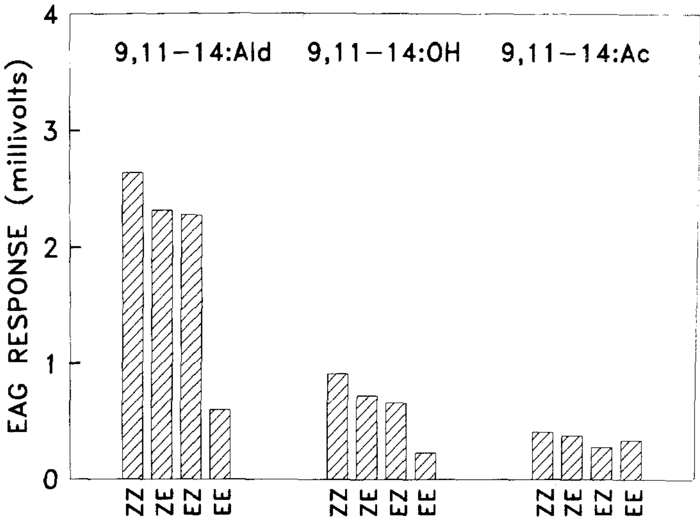


FIG. 1. EAG responses of *L. ferruminaria* males to 9,11-tetradecadienyl aldehyde, alcohol, and acetate geometrical isomers.

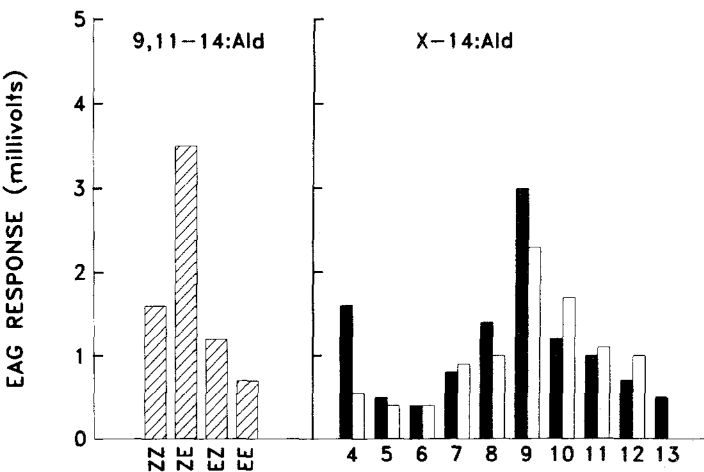


FIG. 2. EAG responses of *D. bilineata* males to the geometrical isomers of 9,11-tetradecadienal and the *Z* (black) and *E* (white) positional isomers of tetradecenal.

responses to *Z*9,*E*11-14: Ald and *Z*-(9)-tetradecenal (*Z*9-14: Ald). The analogous monoenes were not attractive in the field as single components, and they had no significant affect as second components for either *L. ferruminaria* or *D. bilineata*. *D. bilineata* is the only North American species in the Drepanidae family attracted to a synthetic sex attractant. *L. ferruminaria* was not previously

known to range as far northwest as Saskatoon (McGuffin, 1967), and it is the only Geometridae we are aware of that is attracted to conjugated dienes. Bellas et al. (1983) isolated (*E,E*)-9,11-tetradecadienyl acetate from *Epiphyas postvittana* (Walker) and showed it was a pheromone component.

The single-component 8,10-tetradecadiene survey caught an unidentified *Phyllonorycter* sp. (2) (2003 males); the majority were attracted to (*E,E*)-8,10-tetradecadienyl acetate (*E*8,*E*10–14 : Ac) (489 males) or to (*Z,Z*)-8,10-tetradecadienal (*Z*8,*Z*10–14 : Ald) (1090 males). Some other 8,10-tetradecadienes caught fewer numbers, and most of these captures were at different times of the year. Further investigation may show more than one species is being attracted.

The possibility of substitution of dienes for monoenes and vice versa has been a problem in relating sex attractants to actual pheromone components, and some examples of substitution have been noted by Chisholm et al. (1980b), but in all surveys we have undertaken and the literature reviewed, there are no examples of a monoene substituting effectively for a conjugated diene attractant or pheromone. In most cases when an analogous conjugated diene was present with a monoene attractant, the lure was less attractive to that species or trap capture was inhibited totally. When a monoene was added to a conjugated diene attractant, the only significant affect was inhibition of capture (Chisholm et al., 1985; Chisholm and Reed, 1985; Reed and Chisholm, 1985; Reed et al., 1984).

The only example of conjugated dienes substituting for a monoene is the case of *E. ochrogaster* (Table 4) where *Z*5,*Z*7–12 : Ac and *Z*5,*E*7–12 : Ac are substituting as a coattractant. In other cases where combinations of dienes and monoenes are needed for attraction, such as *Phaneta alterana* (Table 1), *Oncocnemis cibalis* (Grote), (Reed et al., 1984), *Cydia flexiloqua* (Heinrich), *Pelochrista scintillana randana* (Kearfott) (Chisholm et al., 1985), no single component was attractive by itself.

More extensive field surveys in other areas will undoubtedly uncover more attractants and probably show more monoene diene interrelationships.

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# DETERMINATION OF CHIRALITY OF ALCOHOL OR LATENT ALCOHOL SEMIOCHEMICALS IN INDIVIDUAL INSECTS

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**Abstract**—A method is described for determining the enantiomeric composition of chiral alcohols, lactones, and hydroxy acids in quantities ranging from 25 ng to 10 μg. Derivatization of the substance with chirally pure acetyl lactate, followed by splitless capillary gas chromatography, enables enantiomeric determinations to be made within 1–3% of the actual value. This technique was applied in the determination of semiochemical in *Ips pini* (Say), *Apis mellifera* (L.), and *Cryptolestes ferrugineus* (Stephens). The results indicate that considerable variability exists within populations of some insects in the composition of their chiral semiochemicals, whereas others produce substances of constant composition.

**Key Words**—Chiral semiochemicals, pheromones, enantiomeric composition, *Ips pini*, Coleoptera, Scolytidae, *Apis mellifera*, Hymenoptera, apidae, *Cryptolestes Ferrugineus*, Cucujidae, acetyl lactate diastereoisomers.

## INTRODUCTION

Many insects utilize the chirality inherent in some semiochemicals as one of a number of means to encode their messages. Determination of chirality has, however, been limited to the sampling of large numbers of insects due to the small amounts of semiochemical present in individual insects. Typically, a large number of insects would be gathered, the semiochemical extracted and purified in a single sample, and the determination of chirality made by either derivative formation, followed by chromatographic (Fish et al., 1984), spectroscopic anal-



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ysis (Plummer et al., 1976), or by optical rotation measurements (Silverstein et al., 1966). If analyses indicated enantiomeric mixtures, very little could be inferred regarding the production of semiochemical within individuals and a question remained as to whether the individuals in that population were uniform or variable in the chirality of the semiochemical they produced.

Splitless capillary gas chromatography (SCGC) permits measurement of small amounts of pheromone present in individual insects due to the high sensitivity and excellent resolving capability of this technique. The direct separation of enantiomers (Schurig et al., 1983) has been achieved but requires the use of chiral capillary columns that are either very expensive or unavailable. Formation of diastereomeric derivatives using chirally pure acetyl lactate (see, for example, Gil-Av and Nurok, 1974) and separation of diastereomers by conventional splitless capillary gas chromatography (Doolittle and Heath, 1984) offers an attractive alternative. We report modifications of this technique that utilize purified reagents and solvents to convert chiral alcohols and their derivatives into separable diastereoisomers, enabling chiral determinations to be made on unpurified extracts of individual insects.

## METHODS AND MATERIALS

*Synthetic Semiochemicals.* The solvents used were analytical reagent grade and were freshly distilled before use. Chiral 2-nonanols were prepared by CuI-catalyzed hexyl Grignard ring opening of chiral methyloxiranes as described for chiral sulcatol, 6-methyl-5-hepten-2-ol (Johnston and Slessor, 1979), and chiral 9-hydroxy-(*E*)-2-decenoic acids were synthesized using Kandil and Slessor's (1983) method. Racemic ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol, was obtained from Borregaard, A.S., Sarpsborg, Norway. Chiral ipsdienol and 11-*S*-(+)-(*Z*)-3-dodecen-11-olide were kindly supplied by E.K. Czyzewska, J.G. Millar, and A.C. Oehlschlager, Department of Chemistry, Simon Fraser University.

*Insect Extracts.* Individual male pine engravers, *Ips pini* (Say), from a laboratory colony established with beetles from the east Kootenay region of British Columbia, were placed on bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, and restrained by gelatin pill capsules (Borden, 1967). After a feeding period of 24–48 hr, beetles were removed from the phloem and their abdomens were excised. Ipsdienol was extracted by placing an individual abdomen in a pentane solution (100 μl) of racemic 3-octanol (internal standard, 4.1 ng/μl) in a 1.9-ml shell vial and macerating it with a microspatula for 15 sec. The extract, as well as a wash (50 μl) with the same 3-octanol solution, was transferred to a 1.9-ml screw-cap vial and placed immediately on Dry Ice. Samples were stored at −70°C.

To obtain 2-nonanol, which has been reported as an alarm substance in the

honeybee, *Apis mellifera ligustica* (L.) (Collins and Blum, 1982), individual foraging workers were chilled to −10°C for 0.5 hr. The ventral side of the abdomen was stroked to expose the sting. The sting and its associated gland were removed with forceps and extracted immediately with an ether solution (10 μl) of racemic 3-decanol (internal standard, 50 ng/μl). The individual extracts were stored on Dry Ice.

9-Hydroxy-(*E*)-2-decenoic acid, a queen-produced honeybee attractant (reviewed by Winston et al., 1982), was extracted from the mandibular glands of mated, individual honeybee queens, *A. mellifera ligustica*, by dissecting and placing the glands in an ether solution (20 μl) containing racemic 3-decanol (internal standard, 50 ng/μl). The glands were gently macerated with a microspatula and the supernatant esterified immediately with boron trifluoride–methanol.

Individual rusty grain beetles, *Cryptolestes ferrugineus* (Stephens), retain insufficient amounts of their lactone pheromones to analyze (Wong et al., 1983). Therefore, pentane extracts of Porapak Q-collected volatiles from feeding adults were obtained from H.D. Pierce, Jr., A.M. Pierce, and A.C. Oehlschlager, Department of Chemistry, Simon Fraser University.

*Acetyl S-Lactyl Chloride Reagent.* *S*-(+)-Lactic acid, L-1750, (Sigma Chemical Company, St. Louis, Missouri) (2.7 g, 30 mmol) was dissolved in freshly distilled acetyl chloride (5 g, 64 mmol) and the solution kept at room temperature for 2 hr. Excess acetyl chloride was evaporated in vacuo, and thionyl chloride (10 g, 84 mmol) was added. The solution was left overnight at room temperature and evaporated to yield crude acetyl lactyl chloride (3.6 g) containing approximately 10% dimeric and trimeric species. Pure monomer (1.2 g) was obtained by careful bulb-to-bulb vacuum distillation on a water aspirator (12 mm, 50–60°C bath temperature). The reagent solution was prepared by dissolving the distillate (25 mg) in dry methylene chloride (1 ml). The reagent solution was stored at 2°C in Teflon-lined screw-cap glass vials and was stable for several months.

*Derivatization of Chiral Alcohols.* Solutions of 2-nonanol in hexane were prepared in various enantiomeric ratios and used as standards. Extracts of *I. pini* containing >25 ng of ipsdienol were concentrated to approximately 20 μl by evaporation at room temperature. Extracts of honeybee sting glands containing >150 ng of 2-nonanol were derivatized directly. To a solution of the standard alcohol (1–10 μg) or an insect extract (10–30 μl) placed in an ampoule prepared from a fresh disposable pipet was added a 50 mg/ml pyridine in ether solution (15 μl) followed by the acetyl *S*-lactyl chloride reagent (30 μl). The components were mixed thoroughly, and the solution was cooled to −20°C and carefully sealed. Ampoules were kept at room temperature overnight, then opened and diluted with hexane (50 μl). The solution was washed by adding water (50 μl), agitated to ensure mixing, and allowed to settle. The aqueous phase was removed, and the organic phase was further washed with aqueous 5%

sodium bicarbonate (3 × 50 μl) and finally once more with water (50 μl). The sample was diluted with hexane to an appropriate concentration for analysis by SCGC. For smaller amounts of alcohol (25–1000 ng), the quantities of the reagents were reduced by one third.

*Derivatization of Hydroxy Acids and Lactones.* Standards, queen mandibular extracts, and rusty grain beetle isolates (0.5–5 μg) were dissolved in methanol (50–100 μl) containing 5% boron trifluoride–etherate, cooled to −20°C, and sealed in glass ampoules. Hydroxy acids were kept overnight at room temperature, whereas lactones were heated at 70°C overnight to convert to the hydroxy ester. Work-up involved dilution with pentane (100 μl) and washing with water (2 × 150 μl, 2 × 50 μl). An aliquot was taken for SCGC analysis, and the remainder was dried over finely divided anhydrous sodium sulfate, concentrated in an air stream, and derivatized as described for alcohols.

*Analyses.* Splitless capillary gas chromatography (SCGC), was carried out on a Hewlett Packard HP 5890 using 30-m × 0.25-mm ID fused silica column, with injector and detector temperatures of 250°C. The column, a methylsilicone DB-1 (J + W Scientific, Inc., Rancho Cordova, California), was temperature programmed as indicated in Table 4. Flame ionization detection was employed with a helium carrier and makeup gas. Kinetic resolution was investigated using several separate derivatizations of racemic 2-nonanol in hexane and working up the samples at intervals.

Analyses of standards and representative and unusual insect derivatives were routinely run on a Hewlett Packard HP 5985B splitless capillary gas chromatograph–mass spectrometer. The mass spectra of the separated diastereomeric derivatives were compared and always found to be nearly identical, disclosing no underlying impurities.

## RESULTS AND DISCUSSION

Analysis of a variety of racemic alcohols by the SCGC acetyl lactate method generally resulted in the baseline separation of the two diastereoisomers with nearly equal intensities, as measured by flame ionization detection. Chirality determination of derivatized 2-nonanol and sulcatol over a range of enantiomeric mixtures were in good agreement with the chirality of the prepared mixtures (Table 1). When chiral mixtures of defined composition were analyzed in five separate experiments, the method proved to be highly reproducible (Table 2). The 3-octanol utilized as an internal standard in the chiral determinations of ipsdienol from *I. pini* was shown to be 51.05% *R*-(−) (SE = 0.15%) for 132 determinations. The presence of only one peak when a pure enantiomer was derivatized indicates the high purity of the lactate reagent as well as the alcohol. Each new preparation of the lactate reagent was checked with a pure chiral alcohol to ensure that partial racemization of the reagent had not occurred during preparation.

TABLE 1. DETERMINATION OF PERCENT $S$-(+)-2-NONANOL AND $S$-(+)-6-METHYL-5-HEPTEN-2-OL IN ENANTIOMERIC MIXTURES BY SCGC OF ACETYL $S$-(+)-LACTYL DIASTEREOISOMERS

| $S$-(+)-enantiomer prepared (%) | 100 ng sample, $S$-(+) enantiomer determined (%) | 900 ng sample, $S$-(+) enantiomer determined (%) |
|---|---|---|
| 2-Nonanol | | |
| 0 | 0.8 | 0.0 |
| 15 | 15.0 | 16.5 |
| 30 | 29.3 | 29.9 |
| 50 | 49.0 | 48.8 |
| 70 | 69.4 | 70.1 |
| 85 | 83.9 | 83.4 |
| 100 | 99.3 | 99.0 |
| 6-Methyl-5-hepten-2-ol | | |
| 0 | 0.9 | 0.6 |
| 15 | 15.1 | 14.0 |
| 30 | 30.1 | 29.0 |
| 50 | 48.7 | 46.9 |
| 70 | 68.8 | 66.4 |
| 85 | 84.6 | 82.5 |
| 100 | 100.0 | 99.4 |

TABLE 2. REPRODUCIBILITY OF CHIRAL DETERMINATION ON TWO SOLUTIONS OF 2-NONANOL: SOLUTION 1, 75% $S$; AND SOLUTION 2, 82% $S$[a]

| Trial | Solution 1, $S$-2-nonanol determined (%) | Solution 2, $S$-2-nonanol determined (%) |
|---|---|---|
| 1 | 74.5 | 84.4 |
| 2 | 74.3 | 81.3 |
| 3 | 74.1 | 82.8 |
| 4 | 73.8 | 82.1 |
| 5 | 74.7 | 81.6 |
| Mean ± SE | 74.3 ± 0.2 | 82.4 ± 0.6 |

[a] Each trial analyzed 0.5-μg samples of the test solutions.

For the simple acyclic alcohols used in this study, there was only minimal kinetic resolution found when derivative formation was incomplete (Table 3). Thus, in the inadvertent case of incomplete reaction, the derivative ratio would deviate less then 5% from the original enantiomeric ratio.

Of the 54 male *I. pini* producing more than 25 ng of ipsdienol, the enantiomeric composition averaged 94% $R$-(−)-ipsdienol, in complete agreement

TABLE 3. KINETIC RESOLUTION OF RACEMIC 2-NONANOL IN INCOMPLETE DERIVATIZATIONS[a]

| Completion (%) | *S*-(+) enantiomer determined (%) |
|---|---|
| 25 | 47.0 |
| 34 | 47.8 |
| 40 | 47.6 |
| 70 | 48.1 |
| 89 | 48.9 |
| 100 | 49.9 |

[a]Reaction begun with 0.5 μg of racemic 2-nonanol and % completion analyzed by SCGC on program a. See footnote *a* Table 4.

with pooled estimates for California (Birch et al., 1980) and Idaho populations (Plummer et al., 1976). However, considerable individual variation occurred in this population (Figure 1). Geographic variation in the production of and response to chiral ipsdienol has been clearly demonstrated for populations of *I. pini* obtained from separate locales (Birch et al., 1980; Lanier et al., 1980). Individual variation in chirality of pheromones, as shown by *I. pini* from a single population, may have profound implications both for population studies and for pest management programs. New populations of insects could arise in response to natural or artificial selection pressures, such as natural catastrophies or pheromone-based mass trapping programs.

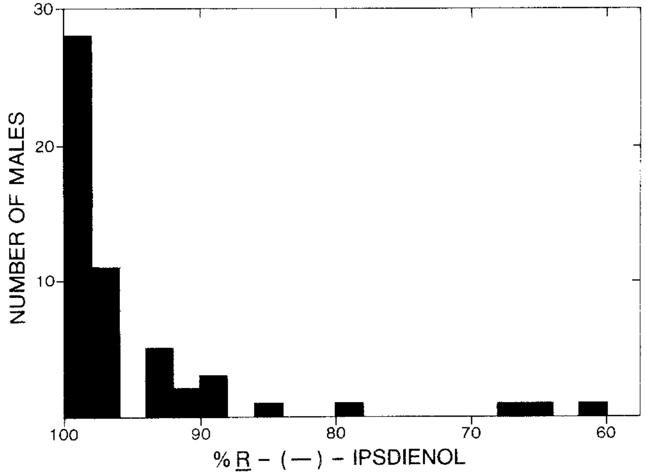


FIG. 1. Chirality of ipsdienol produced by individual male *Ips pini* from the east Kootenay region of British Columbia.

TABLE 4. RETENTION TIMES AND MASS SPECTRAL CHARACTERISTICS OF ACETYL *S*-(+)-LACTYL DIASTEREOMERIC DERIVATIVES

| Compound | Program[a] | | Free alcohol time (min) | Acetyl *S*-lactate time (min) | Predominant mass spectral fragments of *S*-(+)-lactates *m/e* (% abundance) |
|---|---|---|---|---|---|
| *R*-(−)-2-Nonanol | a | | 7.86 | 16.86 | 115(100), 43(91), 71(86), |
| *S*-(+)-2-Nonanol | | | | 17.08 | 88(85), 87(83), 85(60), |
| | | | | | 133(35), 126(15) |
| *R*-(−)-Sulcatol | b | | 5.26 | 15.30 | 95(100), 110(80), 69(47), |
| *S*-(+)-Sulcatol | | | | 15.62 | 43(39), 87(15), 115(10) |
| *S*-(+)-Ipsdienol | c | | 5.92 | 19.30 | 87(100), 43(97), 119(89), |
| *R*-(−)-Ipsdienol | | | | 19.61 | 115(65), 134(65), 91(55), |
| | | | | | 85(55) |
| Methyl *R*-(−)-9-hydroxy-(*E*)-2-decenoate | d | | 19.29 | 37.88 | 81(100), 115(95), 87(84), 123(82), 113(60), 150(50), |
| Methyl *S*-(+)-9-hydroxy-(*E*)-2-decenoate | | | | 38.64 | 133(20), 184(15) |
| 11-*R*-(−)-(*Z*)-3-Dodecen-11-olide | e | Lactone | 15.16 | 29.99 | 81(100), 87(95), 67(85), 55(78), 115(65), 84(62), |
| 11-*S*-(+)-(*Z*)-3-Dodecen-11-olide | | Methyl ester | 19.50 | 30.42 | 95(55), 136(55), 137(45), 210(12) |

[a] SCGC was performed on a 30-m × 0.25-mm ID fused silica methylsilicone (DB-1) capillary column programmed as follows: a, 60°C for 2 min, 7°C/min to 240°C; b, 60°C for 2 min, 7°C/min to 120°C, 2°C/min to 210°C; c, 80°C for 2 min, 10°C/min to 120°C, isothermal for 5 min, 2°C/min to 240°C; d, 60°C for 2 min, 7°C/min to 130°C, 2°C/min to 240°C; and e, 60°C for 2 min, 7°C/min to 180°C, 2°C/min to 210°C.

The chirality of the 9-hydroxy-(*E*)-2-decenoic acid in 22 individual honeybee queens varied from 70 to 95% *R*-(−), with a mean of 84.8±1.8%. These results are in accord with experiments examining behavior elicited by 9-hydroxy-(*E*)-2-decenoic acid, which showed the *R*-(−) enantiomer to be more effective than its antipode in the settling response of queenless honeybee swarms (Winston et al., 1982). Worker honeybees from two hives yielded 2-nonanol with a very high predominance (>95%) of the *S*-(+) enantiomer. The complexity of the semiochemical arsenal of the worker honeybee sting gland (Blum et al., 1978) prevented a more accurate analysis due to overlapping peaks of minor components when analyzed on either column.

The isolates obtained from rusty grain beetle volatiles contained 11-*S*-(*Z*)-3-dodecen-11-olide in greater than 99% enantiomeric purity. This result is entirely consistent with the finding of Wong et al., (1983), that the insects' aggregation response is initiated by the 11-*S* enantiomer.

Our results demonstrate that the chirality of alcohol and latent alcohol semiochemicals can be accurately determined for individual insects. Retention times and characteristic mass spectral fragments of these semiochemical derivatives are reported in Table 4. Determination of the chirality of semiochemical in individual insects will provide a means of examining the variability and monitoring changes in the production of chiral semiochemicals.

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# GENITAL SEX PHEROMONES OF IXODID TICKS: 1. Evidence of Occurrence in Anterior Reproductive Tract of American Dog Tick, *Dermacentor variabilis* (Say) (Acari: Ixodidae)[1]

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**Abstract**—The occurrence of a genital sex pheromone in the anterior reproductive tract of partially fed female *Dermacentor variabilis* was demonstrated by extraction and bioassay. A new type of bioassay, the "neutered" female assay, was developed to test the potency of extracts or chemically defined fractions to stimulate males to copulate. Electrophysiological tests confirmed the ability of males to detect the pheromone with sensilla on their cheliceral digits. Males of both *D. variabilis* and *D. andersoni* exhibited neuronal excitation when stimulated with extracts of the *D. variabilis* reproductive tissues. The pheromone, which is soluble in methanol, was fractionated and found to contain at least two fractions that stimulated copulation by sexually excited males. Evidently, the pheromone is a mixture of two or more compounds. Histologic, ultrastructural, and histochemical studies suggest the vestibular vagina as the site of genital sex pheromone occurrence, presumably from secretions of the surrounding lobular accessory gland. The identity of the compounds that comprise the pheromone remains unknown.

**Key Words**—*Dermacentor variabilis*, Acari, Ixodidae, tick, American dog tick, pheromone, genital sex pheromone.

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## INTRODUCTION

Many hard ticks (Ixodidae) use the sex attractant pheromone 2,6-dichlorophenol to regulate mating behavior, particularly for attracting mate seeking males to sexually ready females (Sonenshine, 1985, review). Often, these species are sympatric and may even share the same hosts at the same time. Frequently, individuals of three, four or even more species can be found on such animals. Remarkably, interspecific matings do not appear to be common (Oliver, 1974), although examples of such events have been noted (Rechav et al., 1982).

To explain the ability of the male ticks to find mates of their own species amidst the mixture of heterospecific and conspecific females, Sonenshine et al. (1982), postulated the existence of a second, or genital, sex pheromone in *Dermacentor variabilis* (Say) and *D. andersoni* Stiles that enables the male to identify its mate and copulate. Using experimental methods, they demonstrated that the males detect a chemical signal on the external genital surface and in the vulva, which enables them to locate the gonopore and deposit their spermatophores. Excited by the nonspecific pheromone 2,6-dichlorophenol, the males visit all females emitting this volatile attractant in order to probe their genitalia; however, they will copulate only with females of their own species. In these species, the males use their chelicerae to locate the gonopore and probe the vulvas of their prospective mates, presumably detecting the genital sex pheromones with chemosensory sensilla on the digits of these appendages. If they fail to detect the appropriate species-specific pheromone, they are unable to form spermatophores and abort the process (Sonenshine et al., 1984). Thus, a multicomponent chemical communication system appears to have evolved to enable pairing of the sexes while minimizing wasteful interspecific matings. Of course, other mechanisms may serve this purpose in different species groups, e.g., the Australian reptile ticks (Andrews and Bull, 1982) or the camel ticks (Khalil et al., 1983).

The source of the genital sex pheromone is unclear and its identity unknown. It may consist of two or more components that stimulate different responses, e.g., probing and spermatophore formation. Moreover, perception of a compound by the animal's sensilla does not necessarily mean that the relevant behavior must automatically follow. Electrophysiological studies are needed to determine what compounds the males perceive in addition to bioassays that measure responses by the act of copulation.

To elucidate the origin and characterize the genital sex pheromone of these ticks, studies were undertaken to extract it from the anterior reproductive tract of sexually active female ticks, *D. variabilis*, and determine the components that excite male sexual activity. Detailed morphologic studies of these organs were undertaken in the expectation that this information might provide evidence of secretory activity consistent with the events surrounding mating. A bioassay

had to be developed in which the putative pheromone was restored to females from which it had been removed, and the ability of males to respond to this artifice determined. The ability of the chemosensilla on the male chelicerae to perceive the pheromone in the female tick extracts also had to be confirmed in order to ensure that these were the organs used in copulation. Finally, chemical methods to fractionate the extracts and isolate the various fractions had to be developed. Following the development of these techniques and specialized studies, it was possible to isolate and study the genital sex pheromone itself.

This paper reports results that demonstrate the existence of a moderately polar multicomponent pheromone in the anterior reproductive tract of the American dog tick, *Dermacentor variabilis*, detected by chemosensilla on the male chelicerae, which stimulates conspecific males to copulate. Chemical studies on the identity of the chemical components that comprise the pheromone will be reported in a subsequent communication.

## METHODS AND MATERIALS

*Ticks.* The American dog tick, *D. variabilis* was colonized with specimens collected near Suffolk, in eastern Virginia. Immature ticks were allowed to feed on albino rats (*Rattus norvegicus*), adults on laboratory rabbits *Oryctalagus cuniculus*. Engorged immatures, unfed stages, and embryonated eggs were held in an AMINCO-AIRE Climate Lab incubator (American Instrument Co., Silver Spring, Maryland) at 27°C ± 1°C and 92% ± 2% relative humidity during the nonparasitic periods of their development. This was the species used for all of the extracts tested, and all of the assays. Adult camel ticks, *Hyalomma dromedarii*, were from a colony originally from the U.S. NAMRU-3 laboratories, Cairo, Egypt (U.S. APHIS permit No. 9433) and were used primarily for cross-mating bioassays, Specimens of the Rocky Mountain wood tick, *Dermacentor andersoni*, were also used for cross-mating bioassays and for eletrophysiological assays. This species was colonized from specimens obtained from the U.S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Montana. Weights of the ticks used in the assays were determined with a Mettler semimicrobalance (Mettler Inst. Co., Cranbury, New Jersey). Female ticks used for the various studies described below were virgin and fed seven days on laboratory rats unless indicated otherwise.

*Scanning Electron Microscopy.* For examination of the anterior reproductive tract (ART) organs with the scanning electron microscope (SEM), female ticks were injected intracoelomically with cold (4°C) 2.5% glutaraldehyde buffered with 0.1 M *s*-collidine or cacodylate (pH 7.4), using a 1-ml tuberculin syringe and a 27-gauge needle. After injection, the ART organs, with the vagina

still attached to the cuticle, were excised and placed in the same cold fixative. The tissues were fixed for 3 hr, washed in 0.1 M *s*-cacodylate, and post-fixed for 2 hr in 1% $OsO_4$ buffered with 0.1 M *s*-cacodylate. The tissues were washed (2×) in buffer to remove excess $OsO_4$, dehydrated through a graded series of ethanol solutions to 100% ethanol, and dried further in a Denton DCP-1 critical point dryer (Denton Vacuum Systems, Cherry Hill, New Jersey) using liquid carbon dioxide. The dehydrated tissues were mounted on aluminum stubs with carbon-conductive paint (E.E. Jeld, Burlington, Vermont) and coated with 100–200 Å of gold–palladium with a sputter coater mounted in a Ladd vacuum evaporator (Ladd Research Industries, Burlington, Vermont). The coated specimens were viewed at 10-kV with a Cambridge Stereoscan model S-100 scanning electron microscope.

*Histology and Transmission Electron Microscopy (TEM).* For histologic study and TEM examination, the ART organs were removed, fixed, and osmicated as described above. Following dehydration in ethanol and 100% acetone, the tissues were infiltrated for 4 hr in 1:1 acetone–Polybed 812, then overnight in 100% Polybed 812, placed in flat molds with fresh epoxy resin, and cured for 48 hr at 65°C. Following hardening, thick (1-μm) and thin (600–800 Å) sections were cut with a Dupont diamond knife using an LKB Ultramicrotome III (LKB Instuments, Inc., Rockville, Maryland). Thick sections were stained for 3 sec in modified methylene blue–azure II (Richardson et al., 1960). The stained specimens were viewed and photographed with a Nikon Optiphot light microscope and camera attachment. Serial sections were prepared as necessary to facilitate recognition of transitions from one organ to another or the entry of ducts into the main channel. Thin sections for TEM study were mounted on uncoated copper grids and stained with saturated uranyl acetate and lead citrate. The stained specimens were viewed using a Hitachi HU-11 B TEM. Measurements of ultrastructural components were made with the aid of an Electronics Graphics calculator (Numonics Corp., Philadelphia, Pennsylvania).

*Histochemistry.* The ART organs were excised, embedded in OCT embedding matrix tissue Tek II (Lab Tek Products, Miles Laboratories, Naperville, Illinois), frozen with Cryokwik (International Equipment Co., Needham Heights, Massachusetts), and sectioned at 6–7 um with an IEC cryostat. The frozen sections were mounted on subbed glass slides. Other specimens were fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned, and stained in accordance with standard histological techniques (Humason, 1972). The following histochemical tests were done in accordance with the methods described by Pearse (1968): oil red O (ORO) for neutral lipids (NL); Millon's reagent for tyrosine-containing proteins; dimethylaminobenzaldehyde (DMAB) nitrite for tryptophan-containing proteins; tetrazonium (TZ) reaction for histidine-, tyrosine-, or tryptophan- containing proteins; Best carmine (BC) reaction for glycogen; and periodic acid–paradiamine (PAD) method for mucopolysaccharides.

*Extracts for Chemical Analysis.* Extracts were prepared by two methods: (1) washing the vulva and external genital cuticle, hereafter termed GW extracts, and (2) excising and collecting the ART organs in methanol, hereafter termed ART extracts. To wash the vulva, a glass micropipette was drawn to a tip diameter of 10–20 $\mu$m with a microelectrode puller inserted into a Leitz microelectrode holder, and connected via Teflon tubing to a glass 50-$\mu$l Hamilton syringe fitted with a 20-gauge needle. The microelectrode holder was mounted onto a Leitz micromanipulator (Bunton Instrument Co., Baltimore, Maryland) to control the movements of the micropipet. To control the volume and rate of flow, the Hamilton syringe was mounted on a Houston Atlas microinjector and set for a flow rate of 0.5 $\mu$l/min. The washing solution was a 50% solution of HPLC grade methanol (Burdick & Jackson Laboratories, Muskegan, Michigan) in distilled water. Each female was flushed 3× and the fluids from many females were accumulated in an ampoule and forwarded to SUNY for chemical study. To prepare the extracts of the ART organs, the ART tissues were excised as described above, including a small (0.5 $mm^2$) area of cuticle surrounding the gonopore, and placed in cold (4°C) HPLC grade methanol. A total of 80, 200, and 700 females were used for ART I, II, and III, respectively, the three extracts which were prepared. Bioassays (see below) were done with aliquots of each crude extract to verify biological activity, whereupon each ampoule containing the ART extracts was flame sealed and forwarded to SUNY, Syracuse, New York for chemical study. Each extract was fractionated (see below) and aliquots of each fraction were returned to Old Dominion University (ODU) for bioassay,

Authentic chemical compounds that might occur in the ART tissues and stimulate copulation were also tested by bioassay (see below). The compounds tested included 20-hydroxyecdysone (20-OH ecdysone), adenosine triphosphate(ATP), and glutathione (GTH) (Sigma Chemical Co., St. Louis, Missouri).

*Bioassay.* The "neutered" female assay was used to measure the response of sexually active males by their ability to copulate with "neutered" females treated with aliquots of the crude extract, chemical fractions of the original crude extracts, or authentic known compounds. This assay is similar to that described by Sonenshine et al. (1982). In this assay, males were exposed to females that had virtually the same external appearance as untreated ticks, but the vagina was severed. To prepare the female for testing, an incision was made in the ventral body cuticle of an unfed fasting specimen, microforceps were inserted, and vagina and uterus were withdrawn. The vestibular vagina was severed, and as much of the vagina and uterus as possible was removed. Surviving females were allowed to feed on a rabbit for seven days, whereupon the partly fed "neutered" females were examined; those found dead, moribund, or exhibiting distortions of the external genital area that would prevent mating were discarded. Next, the rabbit host was tranquilized, and the ventral surfaces of

the remaining "neutered" females were washed (methanol and acetone) and scraped vigorously to remove any residual pheromone. Thus, the "neutered" females presented the same physical appearance externally as the untreated controls except for the appearance of wound scars. Mortality with this technique was found to be ca. 40%. The mean weight of the "neutered" females was 59.9 mg ± 33.6, which was significantly less than the mean weight of the untreated control females, 76.6 mg ± 33.4 ($P < 0.01$, DF).

To perform the assay, an aliquot of a methanolic (or acetonitrile) solution of each crude extract, fraction, or authentic compound was taken up with a micropipet, and ca. 1.0 μl was inoculated into the gonopore with the aid of the Leitz micromanipulator while the attached specimen was viewed with a stereoscopic microscope. The solution was allowed to dry and the treatment repeated until 2–5 μl had been deposited. Some of the solution leaked out and evaporated on the external body surface. The same treatment was applied to a second "neutered" female (paired test, or "trial"). The treated females were allowed to reattach. In some instances, treatments were done with females still attached to the host. Controls were prepared by (1) treating "neutered" females with solvent (methanol) only and (2) not treating partially fed females (not "neutered"). Males were tested with untreated females for demonstration of precopulatory responses; those failing to respond were rejected.

After application of the test solutions, sexually active (SA) males were released adjacent to each female; a limit of five males was used for each female. Males were released until either a male copulated or five males had been tested, whichever came first; each male was allowed three trials to probe and copulate. Males that failed to orient were discarded. Responses were considered positive if the male copulated, as defined by deposition of a spermatophore, or the males probed for an unusually long period of time, in excess of a mean of 10 min. A single test with a pair of treated females and five males for each female represented one trial of a particular extract, fraction, or compound.

To evaluate the bioassay response, a scoring system was developed that gave the greatest weight to copulation by the first males exposed to each female of the test pair, less weight to copulation by one of the subsequent males, and the least value to extended probing behavior. Scores were assigned as follows: 5 = copulations with both females; 4 = copulation with one female, extended probing by males with the other; 3 = copulation with one female, no response with the other; 2 = no copulations, but extended probing behavior by males with both females; 1 = no copulations, but extended probing behavior by the males with one of the two females; 0 = no response. We added 2 points if the male that copulated was the first male, 0.5 points if it was the second male, 0.25 if it was the third, but none if it was the fourth or fifth male. Thus, the maximum attainable score in a single trial was 9 (copulations with both females by the first males in each case). Statistical tests (test of porportions; Steel and Torrie, 1960)

were used to test the significance of the differences between the scores for the treatments versus those of the "neutered" controls.

On completion of the trials, each female was dissected and examined with the stereoscopic microscope to ensure that the vagina had been severed and that the wound scars did not occlude the gonopore. Those females that did not meet these criteria were discarded, and the trials with those test materials were repeated.

To determine whether the solvents may have affected the bioassay responses, aliquots of the crude extract and the different fractions were dried and reconstituted in methanol–Shen's saline (1:1, v/v), implanted, and tested as described above. Controls were observed between the results obtained with the extracts or fractions in this solvent vs. those obtained with methanolic or acetonitrile solutions.

Cross-mating tests were done in the same manner as described for the conspecific tests except that neutered *D. andersoni* or *H. dromedarii* females were substituted for the *D. variabilis* females.

A bioassay of male mating responses to 20-OH ecdysone was also done using the gonopore occlusion method as described by Sonenshine et al. (1982). A total of 15 females were tested with 40 males and 54 mating attempts.

*Electrophysiological Assays.* Standard electrophysiological techniques for recording from contact chemosensilla were employed (Hodgson et al., 1955). Briefly, fed male ticks, *D. variabillis* and *D. andersoni*, were affixed to a metal holder with tacky wax, and the spinose sheaths surrounding the chelicerae were removed with minute needles. The nerves to the capitulum were severed to prevent movement of the chelicerae. Next, a capillary glass microelectrode was drawn with a Narashige microelectrode puller to ca. 10–20 $\mu$m tip diameter, filled with the test solution, and micromanipulated with a Leitz micromanipulator over the sensory area of one of the cheliceral digits. This electrode provided the stimulus and also served as the recording electrode. A Leitz Laborlux compound microscope (704× magnification) was used to view placement of the microelectrode onto the cheliceral digits. The indifferent electrode was an uninsulated tungsten wire, electrolytically etched to ca. 1 $\mu$m tip diameter, inserted into the hemolymph space of the basis capituli, and connected to ground. Details of the preamplifier, audioamplifier, oscilloscope, and recording apparatus were as described by Haggart and Davis (1980). To avoid erroneous interpretations due to electrode contact noise artifacts, the stimulus/recording electrode was frequently placed over nonsensory areas. The chemicals or extracts tested included NaCl at various concentrations, distilled water, ATP (0.5 M), glutathione (0.1 M), 20-OH ecdysone (0.001 M), glucose (1.0 M), the ART extract (crude) dried and reconstituted in distilled water, and the gonopore/vulval washings (GW extract) also dried and reconstituted in distilled water.

*Chemistry.* Upon arrival at SUNY, the ampoules containing an extract were

opened, the solvent decanted, and the solid fragments reextracted with the same solvents (2×); a sonifier (Branson Sonic Power Co., Danbury, Connecticut) was used to enhance membrane disruption and extraction of intracellular compounds. In the case of the first extract (ART I), the original solvent was processed separately (ART I/O) from the tissue fragments, which were sonicated and reextracted as described above. In the case of the other extracts, the solvent and tissues were processed (extracted) and recombined as a single extract. The extracts were separated into fractions by gel permeation, using a (1) Sephadex LH 20-100 gravity feed column, and (2) a Waters Styragel 60 A gel permeation column (size separation 200–2000 amu). A Waters high performance liquid chromatography system, with a model 440 UV detector (254 nm, filter) and model 510 pumps, was used to separate fractions and view any UV-absorbing compounds present. The mobile phase consisted of pure HPLC grade acetonitrile or methanol. In several cases, fractions exhibiting biological activity were purified further by HPLC using a reversed phase Whatman Partasil PXS column 10/25, ODS-2. Aliquots of each fraction were sent to ODU for bioassay. The presence of 20-OH ecdysone in the extracts was confirmed by comparison with an authentic standard (Sigma Chemical Co., St. Louis, Missouri) and by mass spectrometry.

## RESULTS

*Structure.* The female reproductive tract of *D. variabilis* consists of the genital pore, the vestibular vagina (VV), the cervical vagina (CV), the tubular accessory glands (TAG), the connecting tube (CT), the uterus, the receptaculum seminis (RS), and the oviducts. Figure 1 illustrates the organization of the *D. variabilis* female ART. In the unfed ticks, the VV is a broad cuticle-lined duct, ca. 335 $\mu$m long (range 320–360 $\mu$m, $N = 4$) (Figure 2A) covered by a relatively thin membranous epithelium (Figure 2B). When the VV is severed near the genital orifice, the thick, highly folded cuticular lining of this duct, free of the surrounding tissues, becomes evident (Figure 2C).

Histologic examination of this tube in the feeding virgin female reveals the moderately thick (15–20 $\mu$m) highly folded trabecular organization of the VV lining cuticle, with its broad curved lateral edges and the relatively undeveloped epithelium (Figure 2D). At the junction of the vestibular and cervical regions of the vagina are the paired tubular accessory glands (TAG), which extend posteriorly along the dorsolateral edges of the CV and RS (Figure 2A). During feeding (seven days), the surface tissues of the VV poliferate enormously, from ca. 50 $\mu$m to over 160 $\mu$m thick, expanding the trilobed lobular accessory glands (LAG) (Figures 3A and B); near the junction with the CV, additional elongated threadlike and lobular structures appear on the LAG surface (Figure 3C). Cross-sections through the latter region reveal the greatly expanded epithelium, di-

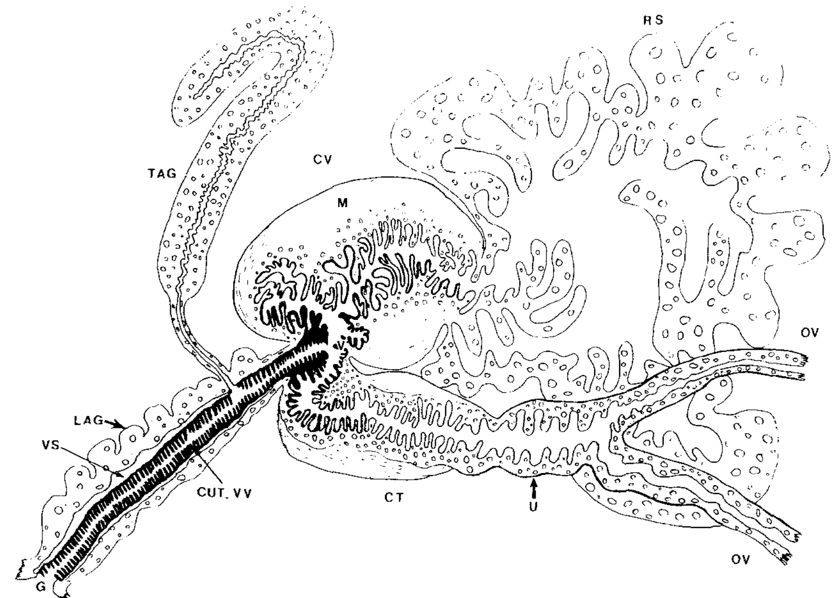


FIG. 1. Stereodiagrammatic representation of the anterior reproductive tract (dorsolateral view) in a feeding, virgin female *Dermacentor variabilis*, illustrating the spatial relationships of the various organs that comprise this system. CV = cervical vagina; CT = connecting tube; G = gonopore; LAG = lobular accessory gland; M = muscle; OV = oviduct; RS = receptaculum seminis; TAG = tubular accessory gland (only one of the pair of glands is figured); U = uterus; V = valve; VS = vestibular sinus; VV = vestibular vagina.

vided by deep grooves into the central and lateral lobes (Figure 3D). The lobes appear to have expanded primarily by cell proliferation since the nuclei are small and close together. In addition, cuticle-lined channels are evident, extending from each of the three lobes of the LAG and emptying into a narrow space, the vestibular sinus, 5–7 $\mu$m wide, visible as a clear zone surrounding the lining cuticle of the VV. This organization of the LAG is also evident in the unfed female, and the channels between the lobes are filled with an unknown material.

When sections of the LAG of the feeding virgin female are examined by TEM, the epithelial cells are found to be rich in mitochondria, numerous ribosomes, and extensive amounts of rough endoplasmic reticulum throughout their cytoplasm; numerous coated vesicles occur near the apical ends of the cells (arrow), and a layer of microvilli occurs near the luminar boundary of the cell membrane with the lining cuticle of the lobular duct (Figure 4A). The organization of the duct cuticle is moderately dense; channels and vacuoles appear surrounded by amorphous granular material interspersed with fibrous material.

The tubular accessory gland consists of a narrow cuticle lined duct and a

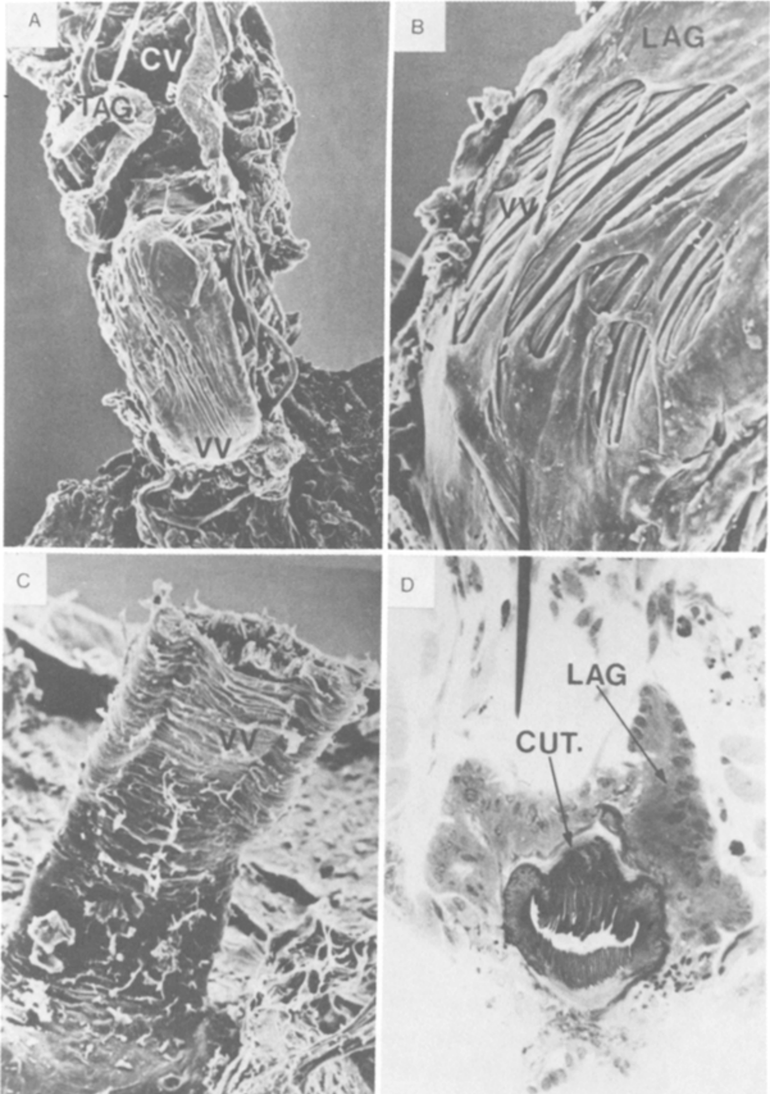


FIG. 2. Scanning electron micrographs of the anterior reproductive tract in an unfed female *D. variabilis*. A. Profile illustrating the vestibular vagina (VV) with a thin, relatively undeveloped epithelial covering, the attachment of the tubular accessory glands (TAG), and the remnant of the cervical vagina (CV) (120×). B. Enlargement illustrating details of the remnant of the epithelium of the undeveloped lobular accessory gland (LAG) epithelium and the exposed cuticle of the duct of the vestibular vagina (VV) (592×). C. Scanning electron micrograph illustrating the remnant of the duct of the vestibular vagina (VV) after it was deliberately severed and the remaining anterior reproductive tract organs removed (337×). D. Light micrograph of a histologic section through the vestibular vagina and surrounding lobular accessory gland (LAG) in an unfed female *D. variabilis*. CUT. = cuticle of VV duct. (350×).

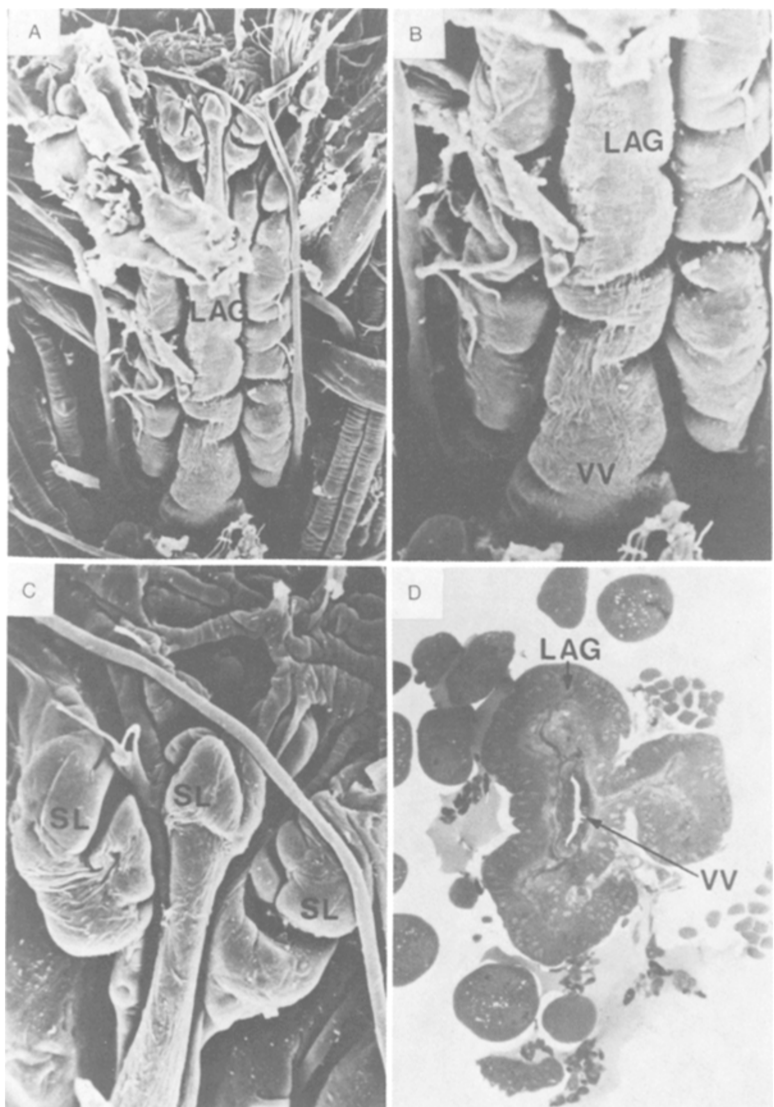


FIG. 3. Scanning electron micrographs illustrating the changes in the anterior reproductive tract during the feeding of the virgin female *D. variabilis*. A. Dorsolateral view illustrating the greatly expanded lobes of the lobular accessory gland (LAG) (290×). B. Enlargement illustrating the swollen three major lobes of the LAG and the attachment of the vestibular vagina (VV) to the gential aperture (580×). C. Enlargement illustrating the swollen lobes of the LAG and the smaller, secondary lobes (SL) near the junction of the vestibular vagina with the cervical vagina (1100×). D. Light micrograph of a histologic section through the vestibular vagina (VV) of a feeding virgin female *D. variabilis*. The lobular accessory gland (LAG) has expanded greatly. The material in the channels from the different lobes fills a large sinus, the vestibular sinus (VS) indicated by the arrow, separating the duct cuticle and the detached lobular epithelium (215×).

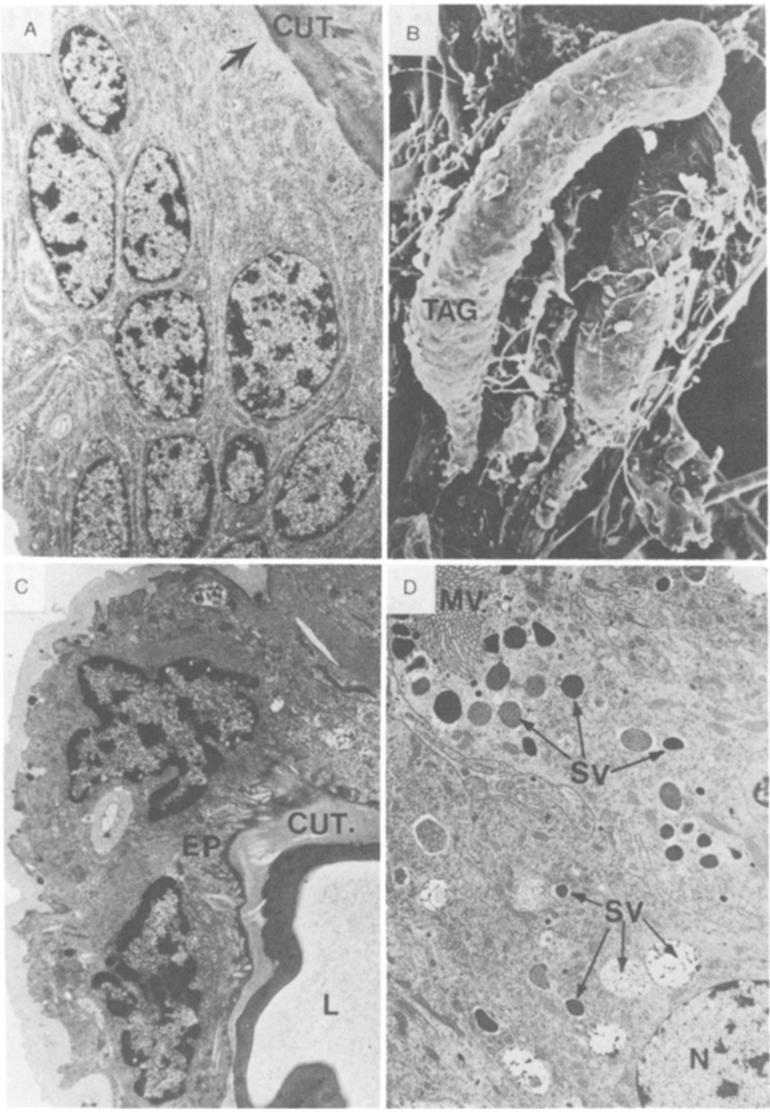


FIG. 4. Transmission and scanning electron micrographs illustrating the ultrastructural characteristics of the epithelium and cuticular lining of the lobular accessory gland (LAG) and the tubular accessory glands (TAG). A. TEM profile of the LAG epithelium illustrating the coated vesicles and microvilli (arrow) near the junction of the cell with the cuticle (CUT.) lining the lobular channel, and the abundant rough endoplasmic reticulum in the cytoplasm of these cells. Numerous ribosomes and mitochondria occur throughout the closely packed cells (1120×). B. SEM micrograph illustrating the swollen secretory area of the tubular accessory glands (TAG) (190×). C. Section through the ductular zone

broad, vermiform glandular region that expands almost threefold during feeding (Figures 1, 2A, and 4B). When viewed with the TEM, the proximal duct is found to consist of a single layer of small epithelial cells lined with a thin (0.3 μm) dense cuticle surrounding a subcircular lumen (Figure 4C). The middle and distal regions are characterized by a multilayered epithelium of large secretory cells and stellate supporting cells. The secretory cells contain masses of secretory vesicles filled with granular material of varying density, some extremely electron dense, others moderately so, while many are reticulate in appearance with little granular content. Membranes, often difficult to discern, appear to surround some of the accumulations of secretory material. Clusters of microvilli occur in the apical regions of the secretory cells (Fig. 4D).

The cervical vagina (CV) is a large, relatively short (by comparison with the VV) tube, with a highly folded lumen surrounded by a multilayered epithelium and three or four layers of smooth muscle cells on its external surface (Figure 5A). It is lined with a well-developed cuticle, ca. 13–25 μm thick, in most areas. When examined with the TEM, the epithelial cells are found to be highly interdigitated (Figure 5B). Microvilli appear at the luminal boundaries of the proximal cells. The cell cytoplasm reveals moderate amounts of mitochondria and ribosomes, but secretory vesicles are absent. The connecting tube (CT) is an extension of the CV, with an extremely folded lumen also lined by a distinct, thin (2 or 3 μm) cuticle (Figure 5C). The epithelial cell profiles resemble those of the CV, although electron-dense granules occasionally do occur. Clusters of microvilli occur at the apical edge of the epithelial cell–cuticle lining interface. The cells are not unusually folded, in contrast to the cervical region. The CT fuses with the uterus on the ventral side of the ART. Posteriorly, the CV fuses with the receptaculum seminis (RS), which forms such of the dorsal–posterior part of the ART (Figures 1, 2A, 5B, and 5D). Histologic sections reveal a collapsed lumen, but not as extensively folded as the CV or CT lumina, with a delicate, thin (ca. 1 μm) cuticular lining. The epithelium is multilayered, with many large cells containing huge nuclei, some in excess of 30 μm. The external surface is lobed. Although some muscle cells are seen on the outer surface of the RS, they do not appear to form an organized muscle layer.

*Experimental Evidence of GSP in the ART.* Bioassays demonstrated evidence of the GSP in extracts of the ART tissues from partially fed *D. variabilis* females (Table 1). The strongest responses were found with the crude extract.

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of the tubular accessory gland. CUT = cuticle; L = lumen; EP = epithelial cell (9360×). D. TEM profile of the epithelial cells of the secretory zone of the TAG, illustrating the types of secretory inclusions prevalent in these cells. Some of the secretory material appears to be surrounded by a membrane. Some vesicles contain densely packed masses of fine granular materials, while others contain few granules in a reticulate pattern; rarely, electron-dense vesicles also occur. MV = microvilli; N = nucleus; SV = secretory vesicles (5180×).

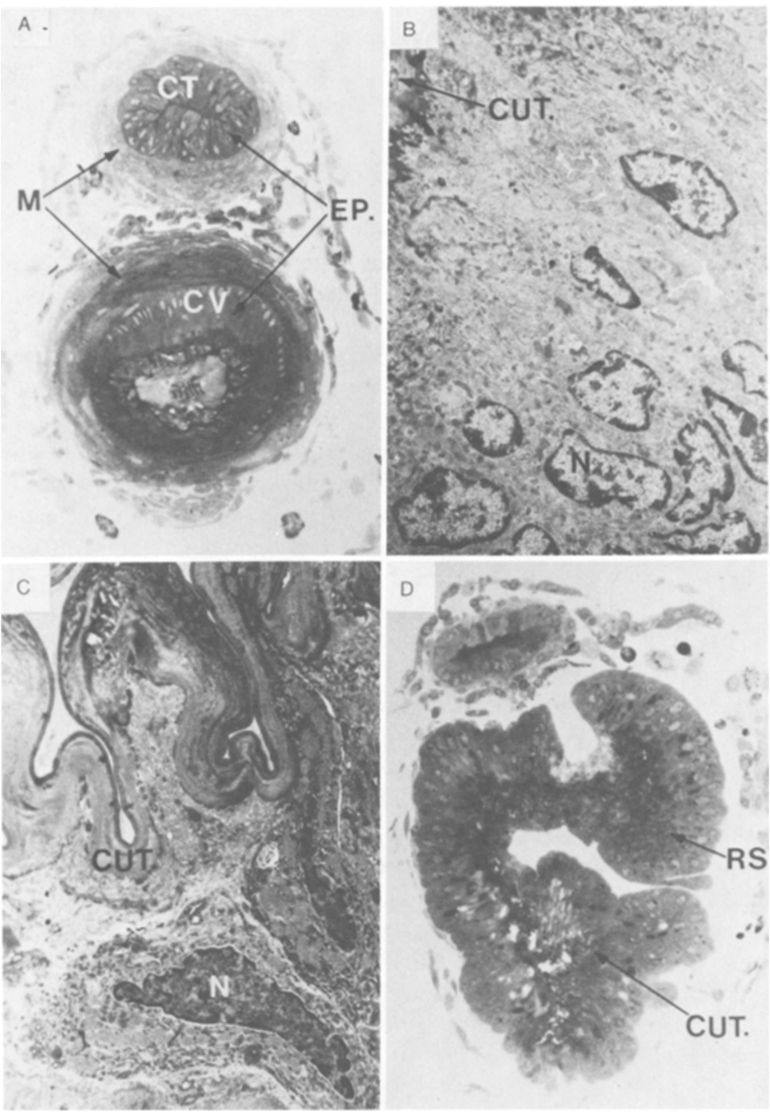


FIG. 5. A. Light micrograph of a cross-section through the cervical vagina (CV) and the connecting tube (CT) showing the distinct separation of the two ducts. Both organs contain layers of smooth muscle surrounding the epithelial layer. EP = epithelial layer; M = muscle layer (100×). B. TEM micrograph illustrating the ultrastructure of the cervical vagina (CV) epithelium. CUT. = cuticle; N = nucleus (3100×). C. TEM micrograph illustrating the ultrastructure of the connecting tube epithelium. CUT. = cuticle; N = nucleus (7840×). D. Light micrograph of a cross-section through the receptaculum seminis (RS) illustrating the collapsed lumen, delicate cuticular lining (CUT.), and lobulate external surface of this organ (100×).

TABLE 1. RESPONSE OF SEXUALLY ACTIVE *Dermacentor variabilis* MALES TO "NEUTERED" FEMALES TREATED WITH CHEMICALLY DEFINED FRACTIONS OF ART EXTRACT[a]

| Extract No. 1 | | | Extract No. 1[b] | | | Extract No. 2 | | |
|---|---|---|---|---|---|---|---|---|
| Extract fraction | Actual score | % | Extract fraction | Actual score | % | Extract fraction | Actual score | % |
| ART I[c] (crude) (0.5 FE) | 7.5 | 83.3** | ART I/2[c] (crude) (0.4 FE) | 7.5 | 83.3** | ART II (crude) (2.0 FE) | 14.0 | 77.8**[d] |
| ART I/1 (4.5 FE) | 1.0 | 11.1 | ART I/2-1 (4.5 FE) | 0.0 | 0.0 | ART II/0 (1.4 FE) | 3.5 | 19.4 |
| ART I/1 (4.5 FE) 2 | 5.3 | 58.3* | ART I/2-1 (4.5 FE) −2 | 3.5 | 38.9 | ART II/0 (1.4 FE) −1 | 0.0 | 0.0 |
| ART I/1 (4.5 FE) −3 | 3.0 | 33.3 | ART I/2-1 (4.5 FE) −3 | 5.3 | 58.3** | ART II/0 (1.4 FE) −2 | 13.0 | 72.2** |
| ART I/1 (4.5 FE) −4 | 5.0 | 55.6** | ART I/2-1 (4.5 FE) −4 | 9.0 | 100.0** | ART II/0 (1.4 FE) −3 | 4.3 | 23.9 |
| ART I/1 (4.5 FE) −5 | 6.0 | 66.7** | ART I/2-1 (4.5 FE) −5 | 0.0 | 0.0 | ART II/0 (1.4 FE) −4 | 6.3 | 35.0 |
| ART I/1 (4.5 FE) −6 | 3.0 | 33.3 | ART I/2-1 (4.5 FE) −6 | 5.0 | 55.6* | ART II/0 (1.4 FE) −5 | 3.5 | 19.4 |
| ART I/1 (4.5 FE) −7 | 0.0 | 0.0 | Recomb. | 7.5 | 83.3** | ART II/0 (1.4 FE) −6 | 9.3 | 51.7** |
| Recomb. | 1.0 | 11.1 | | | | ART II/0 (1.4 FE) −7 | 10.0 | 55.6* |
| | | | | | | ART II/0 (1.4 FE) −8 | 10.5 | 58.3** |
| | | | | | | ART II/0 (1.4 FE) −9 | 3.5 | 19.4 |
| | | | | | | Recomb. (1.0 FE) | 4.5 | 25.0 |

[a] Explanation of scoring system: 0, no copulations, males probed female gonopores < 10 min; 1, no copulations, 1 male probed > 10 min; 2, no copulations, but both males probed > 10 min; 3, 1 male copulated, other male probed > 10 min; 4, 1 male copulated, other male probed > 10 min; 5, both males copulated. Copulation is defined as deposition of a spermatophore. Add 2 points if the male that copulated was the first male, 0.5 points if it was the second male, 0.25 points if it was the third male, 0 points if it was the 4th or 5th male. Example: ART I (crude), in the first trial, copulation occurred with the first male on 1 female, no copulations any of the 5 males tested on the other female (score = 5); in the second trial, copulations occurred with the first male on both females (score = 9), final score = 14. Frequency of copulations with controls (untreated, neutered females) was 23.7% in 13 trials containing 26 females and 117 males (319 trials).

[b] Reextraction of particulates.

[c] One trial with 2 treated females, total possible score/treatment = 9.

[d] Two trials with 2 treated females/trial, total possible score/treatment = 18. * = positive $T$ test (test of proportions; Steel and Torrie, 1960), $P < 0.01$; ** = positive $T$ (test of proportions; Steel and Torrie, 1960), $P < 0.001$.

Strong positive responses were also obtained with several of the fractions isolated by gel permeation chromatography. Fraction numbers are assigned in order of decreasing molecular weight. Significant positive responses (denoted by asterisks) were obtained with fractions 2, 4, and 5 in ART I; fractions 3, 4 and 6 in ART I/O; and fractions 2, 6, 7, and 8 in ART II. The responses to fraction 4 in ART I/O and fractions 2 in ART II were not significantly different than the responses obtained with the crude extracts in each case ($T = 1.27$, 12 *df*, NS; $T = 0.51$, 61 *df*, NS; respectively). In ART I, positive responses were obtained with fractions containing molecules in the range of 600–1000 amu (fraction 2) and in the range of 400–500 amu (fractions 4 and 5). In ART I/O, positive responses were obtained in the range of 600–1000 amu (fractions 3 and 4) and 350–500 amu (fraction 6). In ART II, an extremely strong positive response was obtained with a fraction with molecules in the range of 600–1000 amu (fraction 2); positive responses were also obtained with fractions containing molecules in the range 380–500 amu (fractions 6, 7, and 8). Recombining the fractions led to a positive response in only one of the three extracts assayed (ART I/O).

No response was observed with bioassays of the gonopore/vaginal washings (GW extract) using the "neutered" female bioassay (8 females, 40 males, 120 male mating attempts).

The bioassays were repeated with a third extract, ART III, comprising material from the anterior reproductive tract of 700 *D. variabilis* females. A total of 16 fractions were isolated by gel permeation and collected for assay; in addition, a sample of 20-hydroxyecdysone was included (fraction 17) as a blind study. Finally, the extract was recombined with aliquots of each fraction. The results of the bioassays are summarized in Table 2. Strongly positive responses were obtained with fractions 2 and 3 (containing molecules in the range of 600–1000 amu), 7 (estimated molecular size ca. 500–700 amu), 13 and 14 (containing molecules in the range of 400–500 amu), and with the sample of 20-OH ecdysone, fraction 17.

Tests were also done with authentic, known compounds. Table 3 summarizes the results of tests with the hormone, 20-OH ecdysone, ATP, and GTH. Tests were done with different amounts of 20-OH ecdysone, as well as a fraction from the tick extrct, ART II fraction 5 containing this compound. No response was found with 0.01 $\mu$g of 20-OH ecdysone, but strong positive responses were obtained when the amount was increased to 0.1 and 2.0 $\mu$g. No significant response was obtained with the 20-OH ecdysone-containing fraction from the tick extract, although one copulation was noted and the percent response was increased when compared with the controls. No significant responses were obtained with ATP or glutathione, and no copulations were observed.

In addition to the tests done by means of the "neutered" female bioassay, a single test of the male response to 20-OH ecdysone using the gonopore occlusion method gave indications of male response to this compound (not shown in

TABLE 2. SUMMARY OF BIOASSAY RESPONSES BY *D. variabilis* MALES TO COMPONENTS OF EXTRACT ART III, USING "NEUTERED" FEMALE BIOASSAY[a]

| Extract fraction | FE | Trial No. 1 | | Trial No. 2 | | SUMMARY | | Signif. |
|---|---|---|---|---|---|---|---|---|
| | | Raw score | Response (%) | Raw score | Response (%) | Raw score | Response (%) | |
| Crude | 5.7 | 7.0 | 77.8 | — | — | 7.0 | 77.8 | ** |
| 1 | 5.7 | 0.0 | 0.0 | 3.0 | 33.3 | 3.0 | 16.7 | |
| 2 | 5.7 | 5.0 | 55.6 | 9.0 | 100.0 | 14.0 | 77.8 | ** |
| 3 | 5.7 | 9.0 | 100.0 | 7.0 | 77.8 | 16.0 | 88.9 | ** |
| 4 | 5.7 | 5.0 | 55.6 | 3.5 | 38.9 | 8.5 | 47.2 | * |
| 5 | 5.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| 6 | 5.7 | 7.0 | 77.8 | 0.0 | 0.0 | 7.0 | 38.9 | |
| 7 | 5.7 | 5.0 | 55.6 | 7.0 | 77.8 | 14.0 | 77.8 | ** |
| 8 | 5.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |
| 9 | 5.7 | 3.3 | 33.3 | 1.0 | 11.1 | 4.0 | 22.2 | |
| 10 | 5.7 | 5.0 | 55.6 | 1.0 | 11.1 | 6.0 | 33.3 | |
| 11 | 5.7 | 0.0 | 0.0 | 5.0 | 55.6 | 5.0 | 27.8 | |
| 12 | 5.7 | 0.0 | 0.0 | 5.0 | 55.6 | 5.0 | 27.8 | |
| 13 | 5.7 | 9.0 | 100.0 | 4.3 | 47.8 | 13.3 | 73.9 | ** |
| 14 | 5.7 | 5.0 | 55.6 | 5.0 | 55.6 | 10.0 | 55.6 | ** |
| 15 | 5.7 | 3.0 | 33.3 | 5.0 | 55.6 | 8.0 | 44.4 | * |
| 16 | 5.7 | 3.5 | 38.8 | 1.0 | 11.1 | 4.5 | 25.0 | |
| 17 | 5.7 | 7.3 | 80.6 | 5.8 | 64.4 | 13.1 | 72.8 | ** |
| Recomb. | 5.7 | 3.0 | 36.7 | 3.3 | 33.3 | 6.3 | 35.0 | |

[a] See Table 1 for explanation of scoring system.

TABLE 3. RESPONSES OF SEXUALLY ACTIVE MALE *Dermacentor variabilis* TO "NEUTERED" FEMALES TREATED WITH AUTHENTIC KNOWN COMPOUNDS OR KNOWN COMPOUND FOUND IN ART EXTRACT[a]

| Treatment | Conc. (μg) | No. trials | Summary score | Response (%) |
|---|---|---|---|---|
| 20-OH Ecd | 0.01 | 1 | 0.0 | 0.0 |
| 20-OH Ecd | 0.1 | 1 | 7.5 | 83.3**[b] |
| 20-OH Ecd | 2.0 | 2 | 10.8 | 59.8 |
| 20-OH Ecd | ART II-5 (?) | 1 | 3.5 | 38.9 |
| ATP | 25.0 | 2 | 3.3 | 18.3 |
| Glutathione | 30.0 | 2 | 0.0 | 0.0 |
| "Neutered" controls | | 13 | 27.8 | 23.7 |

[a] Explanation of scoring system as in Table 1.
[b] *Significant at $P < 0.02$; **significant at $P < 0.01$.

the tables). One male deposited a spermatophore on top of the lacquer barrier; five others cut, tore, or fragmented the lacquer with their chelicerae, even though they failed to deposit spermatophores.

In addition to the tests with neutered females of the same species, tests were also done with the *D. variabilis* extract (ART III) implanted into the vulval remnants of neutered *H. dromedarii* and *D. andersoni* females. Authentic 20-OH ecdysone and ecdysone were also implanted in amounts ranging from 0.25 to 2.0 μg (0.5–2.6 × $10^{-3}$ M for 20-OH ecdysone, 1.1–4.3 × $10^{-3}$ M for ecdysone). Bioassays were done with sexually active *D. variabilis* males. The results are summarized in Tables 4 and 5. Males responded positively (with copulation) to the neutered *H. dromedarii* females treated with the crude extract, but only rarely with the neutered controls. Although significant, the response was much less vigorous than that observed with conspecific females. A positive response was also obtained with fraction 4 and one concentration of 20-OH ecdysone; no response was observed with implants of ecdysone.

*Electrophysiological Assays.* Chemosensory responses were obtained from the inner cheliceral digits of partially fed male *D. variabilis* and *D. andersoni*. No responses were obtained from the outer cheliceral digits of either species. In all cases, the responses were characterized by an increase in overall level of nerve spike activity, with amplitudes varying from 25 to 50 μV. It was impossible to discriminate between the activities of individual neurons based on amplitude. Consequently, we used the summed multiple unit activity of all neurons responding to the stimulus as an indicator of response intensity. The magnitude of the response, therefore, represents the sum of both the number of neurons responding and the discharge frequencies of the individual neurons (Figure 6).

TABLE 4. SUMMARY OF BIOASSAY RESULTS: TESTS OF MATING RESPONSES OF *D. variabilis* MALES TO *H. dromedarii* FEMALES TREATED WITH ART III EXTRACT COMPONENTS AND AUTHENTIC COMPOUNDS[a]

| Extract or compound | Amount or FE applied | Raw score | Percent Response (%) (significance) |
|---|---|---|---|
| ART III (crude)[b] | 11.4 FE | 7.75 | 43.1 ($P < 0.01$) |
| ART III Fract. 2 | 14.0 | 1.3 | 14.1 |
| ART III Fract. 3 | 14.0 | 0.0 | 0.0 |
| ART III Fract. 4 | 14.0 | 2.1 | 23.2 |
| ART III Fract. 13 | 14.0 | 0.0 | 0.0 |
| ART III Fract. 17 | 14.0 | 0.0 | 0.0 |
| ART III Recomb. | 14.0 | 0.0 | 0.0 |
| 20-OH ecdysone | 0.25 μg | 0.0 | 0.0 |
| 20-OH ecdysone[b] | 0.50 μg | 4.0 | 22.2 ($P \leq 0.05$) |
| 20-OH ecdysone[b] | 1.0 μg | 1.0 | 5.6 |
| 20-OH ecdysone[b] | 1.25 μg | 0.6 | 7.1 |
| Ecdysone | 0.5 μg | 0.0 | 0.0 |
| Ecdysone | 1.0 μg | 0.0 | 0.0 |
| Ecdysone | 1.25 μg | 0.0 | 0.0 |
| Ecdysone | 2.0 μg | 0.0 | 0.0 |
| Control[c] | — | 15.75 | 10.6 |

[a]Each treatment tested with 2 "neutered" females and 5 males/female, and 3 trials/male, except when indicated otherwise. The theoretical maximum score that could have been obtained is 9.0

[b]Each treatment tested with 4 "neutered" females and 5 males/female, and 3 trials/male. The theoretical maximum score that could have been obtained is 18.

[c]A total of 33 "neutered" females were tested with 5 males/female, and 3 trials/male, for a total of 495 trials. The theoretical maximum score that could have been obtained is 148.

TABLE 5. RESULTS OF CROSS-SPECIES TESTS TO EVALUATE RESPONSE OF *D. variabilis* MALES TO 20-OH ECDYSONE IMPLANTED INTO FEMALES OF *D. andersoni*[a]

| Treatment | Amount | Actual score | % | No. copul. | No. females | No. trials | No. Males |
|---|---|---|---|---|---|---|---|
| *D. variabilis* males | | | | | | | |
| 20-OH ecdysone | 0.5 μg ($1 \times 10^{-3}$ M) | 1.2 | 13.6[b] | 2 | 13 | 175 | 59 |
| *D. andersoni* males | | | | | | | |
| 20-OH ecdysone | 0.5 μg ($1 \times 10^{-3}$ M) | 1.37 | 15.3[b] | 2 | 12 | 159 | 54 |
| Control (neutered, but w/o hormone) | — | 1.07 | 11.9 | 3 | 28 | 153 | 53 |

[a]Scoring system same as in Table 1.

[b]Not significantly different from controls.

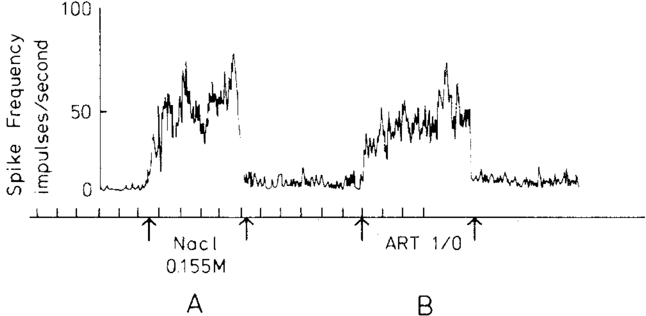


FIG. 6. Instantaneous spike frequency record (1/interval between spikes) of chemosensory neuronal activity in response to specific chemical stimuli applied to the cheliceral digits of *D. variabilis* and *D. andersoni* males (part fed); vertical arrow = stimulus on, curved arrow = stimulus off. (A) Response to 1 M NaCl; (b) response to the extract ART I/O.

The responses of the male cheliceral digit sensilla to the various chemical stimuli presented are summarized in Table 6. We observed concentration-dependent increases in multiple-unit spike activity to NaCl solutions over the range from $2 \times 10^{-2}$ M to 1.0 M; a weak ambiguous response was observed at $1 \times 10^{-2}$. We also obtained moderate to strong positive responses to the ART crude extracts and strong positive responses to the gonopore wash (GW) extract with males of both species. We also obtained positive responses to ATP (0.5 M), glutathione (0.1 M), and 20-OH ecdysone. However, no increase in spike frequency was observed when cheliceral digits of either species were tested with distilled water or 1.0 M glucose solutions. No response was obtained when the pipet microelectrode was applied to presumed nonsensory areas of the capitulum, with one exception.

*Histochemical Results.* The LAG gave a strong positive response to the BC reagent for glycogen, while selected cells and the material in the LAG ducts gave a moderate response with ORO. The ORO reaction was diffuse in some areas, rather than the numerous tiny droplets typical of neutral fats, but other areas of the gland showed numerous intensely staining droplets of varying sizes; staining was also found in the channels of this gland. Strong ORO responses were found with the hemocytes and diffuse fat body cells adhering to the surface of the LAG, RS, and CT (with tiny droplet reactions typical of neutral fats), but these cells are external to the reproductive organs. The cuticular lining of the VV exhibited a diffuse staining reaction with ORO; a strong positive reaction with Millon's reagent, indicating the presence of tyrosine containing proteins; and a dark blue color with the PAD reagent, indicating presence of the mucopolysaccharides; no response was found with any of the other reagents used. The TAG gave a strong positive response to the TZ reagent for histidine-, tyrosine-, or

TABLE 6. ELECTROPHYSIOLOGICAL RESPONSES OF CHEMOSENSORY SENSILLA ON CHELICERAL DIGITS OF SEXUALLY ACTIVE MALES AND TO EXTRACTS OF FEMALE TICK REPRODUCTIVE TISSUES AND OTHER STIMULI

| Type of stimulus | Responses with species tested[a] | |
|---|---|---|
| | *D. variabilis* | *D. andersoni* |
| ART I (crude) | NT | + + + |
| ART I/2 (crude) | + + | NT |
| GW extract | + + + | + + + |
| NACL | | |
| 0.01 M | + | NT |
| 0.16 M (0.9%) | NT | + + + |
| 0.02 M (0.1%) | NT | + + |
| 0.1 M | + + | NT |
| 1.0 M | + + + | NT |
| Dist. $H_2O$ | 0 | NT |
| ATP 0.5 M | NT | + + |
| Glutathione (0.1 M) | NT | + |
| 20-OH ecdysone ($1 \times 10^{-3}$ M) | NT | + + |
| Glucose, 1.0 M | NT | 0 |
| Nonsensory area | 0 to + +[b] | NT |

[a] Explanation of responses: + + + = strong, + + = moderate, and + = weak increase in activity; 0 = no change in activity; NT = not tested. All responses are net, i.e., after consideration of any noise induced during electrode contact (nonsensory area and artifacts).

[b] Most tests of nonsensory areas were negative, but increases to as high as + + were observed in several instances.

tryptophan-containing proteins, but not to any of the other reagents used. The CV and CT cuticle reacted strongly with the BC and PAD reagents, while the muscle layers of these organs reacted with the TZ reagent. The epithelium of the uterus and oviducts reacted strongly to the BC reagent. No other reactions with any of the reagents used were observed.

*Chemical Constituents of ART Extract.* The presence of 20-OH ecdysone was confirmed by coelution with an authentic standard analyzed on the reversed phase column (HPLC) and verified by mass spectrometry. The presence of cholesterol was confirmed by mass spectrometry.

## DISCUSSION

The credibility of the evidence of the GSP, both in the crude extracts and in the fractions, is related to the credibility of the assays for detecting it. The "neutered" female assay assumes that if the GSP is real, it should be possible to (1) remove it and thereby destroy the response, and (2) restore it and thereby

restore the response. The assay is based on the observations of Sonenshine et al. (1982), who demonstrated that males would not copulate with females that had the vaginal duct severed and gential surfaces washed. However, their test sample size was limited (10 female *D. variabilis* and 9 female *D. andersoni*). When large samples are assayed, some copulations occur. Thus, the "neutering" procedure is not 100% effective. When the tissues of the "neutered" females were examined with SEM, a considerable length of the original VV duct was found attached to the gonopore cuticle (up to 185 $\mu$m, or ca. 55% of the original length), despite attempts to remove more of it. This ductular remnant is important since this is the structure into which the male inserts its chelicerae (Feldman-Muhsam, 1971; Feldman-Muhsam and Borut, 1971) and detects the chemical stimuli. The GSP may also be present in unfed females. In previous studies with *D. variabilis* (Sonenshine et al., 1982), 14.6% of male mating attempts with attached but unfed females (attached 3 hr) were successful. Presumably, additional pheromone is synthesized during feeding. Thus, some females may be expected to retain residual GSP, despite efforts to remove it, and some copulations will occur. However, extensive testing ("neutered" female controls, Table 1) indicates that this event occurs in less than 25% of the trials. With further refinement, e.g., screening all "neutered" females prior to treatment with extracts or chemicals, it is possible to reduce this frequency even further. Of course, other types of assays may be considered, and the development of other, perhaps even more suitable methods for study of GSP is not precluded. In our experience, however, attempts to develop assays with two other methods (the gonopore occlusion technique and the transspecific mating technique) were unsuccessful, the former due to difficulties in securing the blockage of the opening, the latter due to difficulties in stimulating consistent male mating responses (Sonenshine and Taylor, unpublished).

We believe that the "neutered" female bioassay meets the criteria set forth in the previous paragraph. Neutering reduced the male mating response to less than 25% of the matings attempted; adding the crude extracts of the ART tissues restored the response, elevating it to an average of 80.6%, which is not significantly different from that observed with "normal" (i.e., untreated) females. The fact that the crude extract gave the maximum response provides additional support for the value of this assay. The only discrepancy is the absence of consistently strong positive response with the recombined extract, a fact that we cannot readily explain.

The strong positive response of sexually active males to heterospecific females treated with the conspecific extract provides additional evidence of the chemical nature of copulation-eliciting stimulus (i.e., genital sex pheromone). In effect, this test meets the criterion set forth in the previous paragraph even more effectively than with conspecific females. Normally, *D. variabilis* males do not mate with *H. dromedarii* females (Khalil et al., 1983). Therefore, to elicit copulation, it was necessary to remove natural components of the vagina

and substitute those of the conspecific female, i.e., *D. variabilis*. That this test succeeded provides convincing evidence of a component that can be removed and restored, i.e., a pheromone.

Electrophysiological assays provide evidence implicating a GSP in the ART tissues using a totally different method than the bioassay. The strong responses observed are especially significant when one considers that the extraction procedures used would retain little NaCl, ATP, or GTH, substances which are known stimuli for the cheliceral digit sensilla (Waladde and Rice, 1977, 1982). Of course, the male neuronal responses must be viewed in the context of all of the evidence. GSP is not the only possible material in the extract capable of stimulating these neurons. Indeed, an extensive gustatory capability may be inferred in view of the discovery of chemosensory neurons innervating the cheliceral digit sensilla (Sonenshine et al., 1984; Waladde and Rice, 1977, 1982). However, these findings do at least demonstrate that the males are able to perceive substances contained in these extracts with their chelicerae. Of special interest is the finding of a positive response to 20-OH ecdysone, the same compound found to be stimulatory in the bioassays (Table 2). Also of interest is the finding that *D. variabilis* and *D. andersoni* males both detect the gonopore/vulval washings (GW extract). Of course, perception need not necessarily lead to a response; none of the males copulated with females treated with the GW extract in the bioassays.

In summary, evidence obtained by two different types of assay methods indicates the presence of chemical substances in the female ART tissues that can be perceived by the males and that stimulate these males to copulate. This evidence, plus the evidence of a removable aphrodisiac on the genital surface (Sonenshine et al., 1984) and the need for a female-originated stimulus to excite the male to commence spermatophore formation, argues persuasively for the existence of a GSP in these ticks, originating in the reproductive organs.

If the credibility of the assays and the existence of GSP is accepted, the results of the tests are also credible and may be evaluated. The identity of the pheromonal components is unknown. Clearly, they are not ATP or GTH, compounds which the ticks are capable of detecting but to which they do not respond by copulation. Considerable interest attends the responses to the hormone 20-OH ecdysone. This compound, which occurs in *D. variabilis* (Dees et al., 1984) as well as in other tick species (Solomon et al., 1982, review), was also found in the ART tissues (Brossut, unpublished). Males responded strongly when this compound was administered in conspecific tests, but not in cross-mating tests (one exception).

Although the responses to 20-OH ecdysone merit attention, we do not conclude that this compound is the genital sex pheromone. If this were so, one would have expected a more consistent response in the cross-mating tests. Moreover, in view of its widespread occurrence in ticks, it could hardly serve as a specific excitant. However, it may serve as a pheromone component in *D.*

*variabilis* so that, when combined with one or more other stimulatory molecules, *D. variabilis* males are induced to form and implant their spermatophores, i.e., to copulate. Our electrophysiological evidence indicates that males are capable of detecting it, which is not unexpected in view of reports of ecdysteroid excitation of neuronal activity (Koolman and Spindler, 1983). When applied to the lacquer barrier covering the gential aperture in the gonopore occlusion test, males cut or tore at the lacquer and one even deposited a spermatophore. There can be little doubt that this compound is excitatory for this species in some way.

In so far as specificity for *D. variabilis* is concerned, the hormone appears to be unusually abundant in the ART of *D. variabilis*, although this may reflect hemolymph contamination on the surface of the tissues rather than actual occurrence in the tissues and ducts of this organ system. Sonenshine (unpublished) observed a 15-fold greater concentration of ecdysteroids in the ART of *D. variabilis* females than in the ART of *H. dromedarii* females when extracts of both species were assayed by radioimmunoassay. Consequently, it is possible that in neutered conspecific females where traces of the natural pheromone may remain in the vulval remnant, the addition of 20-OH ecdysone may have provided sufficient stimulation for the males to recognize the traces of other stimulatory molecules still present and to copulate. In the cross-mating tests, except for one marginally positive response with 20-OH ecdysone ($P \leq 0.05$), only the crude extract gave a vigorous positive response. In summary, we suggest that 20-OH ecdysone may serve a unique role in *D. variabilis* as a contributory component of a complex pheromone. The identity of other molecules, particularly those in the range of 600–1000 amu that elicit the most vigorous excitatory responses, is unknown.

The source of the GSP in the ART organs is also unclear. The most likely candidate is the LAG. This gland, which surrounds the VV and expands greatly during feeding, secretes an unknown substance into the channels that fill the space around the cuticular lining of the VV duct. These channels and fluid layer around the VV create a fragile structure that tends to fragment when the tissues are prepared for histologic study, leading to the impression that the gland detaches and separates from the VV (Balashov, 1972). The cells do detach, but only as a result of the fluid filled channel between the gland and the duct. Lees and Beament (1948) note that the histologic appearance of the epithelium of this gland in *Ixodes ricinus* is remarkably similar to that of Gene's organ, and they conclude, on that basis, that the LAG "is responsible for secreting the wax." Diehl et al. (1982) suggest that the LAG discharges a lipid-rich secretion which penetrates the VV cuticle, entering the duct lumen and coating the eggs. These events occur during oviposition. Less attention, if any, has been given to the LAG during feeding of the virgin female. During this period, the gland has already enlarged and has produced lipids (including steroids?). Of special interest is the diffuse ORO-positive reaction of the VV cuticle, which may indicate transport of secretions through this lining. The ultrastructure of the VV cuticle,

with its numerous cavities, channels, and extremely folded lining, suggests a system that might facilitate transport of secretory substances into the lumen. Bioassays of the LAG region were positive, but that does not necessarily imply that this gland is the GSP gland, since substances made elsewhere could have been transported into the VV. Another candidate is the TAG, which expands during feeding and exhibits clear evidence of secretory activity. However, bioassays of the TAG were negative, and histochemical tests suggest that the secretory substances seen in the secretory vesicles (Figure 4D) are proteinaceous, as suggested by Balashov (1972). Diehl et al., (1982) state that these glands secrete a protein-rich secretion that coats the egg as it passes through the vagina; there is no evidence to implicate the secretions in the formation of the GSP. The histologic and ultrastructural characteristics of the remaining ART organs, the CV, CT, uterus, and RS, do not suggest glandular activity in the feeding virgin female, although the RS becomes secretory after fertilization (Raikhel, 1983).

The chemical composition of the ART extracts will be described in a future paper.

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# DISTURBANCE PHEROMONES IN THE CRAYFISH *Orconectes virilis*

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**Abstract**—The reactions of individual crayfish to the introduction of waters from tanks containing other individuals were recorded to test for the release of chemicals by stressed crayfish. Female *Orconectes virilis* and male *O. rusticus* did not show responses to stressed crayfish. Male *O. virilis* responded differently to undisturbed and disturbed male crayfish (conspecific and heterospecific). Responses to waters from tanks which contained disturbed individuals were similar whether the source of disturbance was aggressive, predatory, or thermal. Chemical(s) involved appear to persist for at least one hour at room temperature.

**Key Words**—Crayfish, *Orconectes virilis*, Crustacea, pheromones, stress, heterospecific responses, threshold effects, predation, thermal stress.

## INTRODUCTION

Alarm pheromones and Schreckstoff are widely known in aquatic systems (Pfeiffer, 1963; Sleeper et al., 1980). In crayfish, reactions to water in which conspecific males were aggressively interacting have been reported for both *Procambarus acutus* (Thorp and Ammerman, 1978) and *Orconectes virilis* (Hazlett, 1985).

The possibility was raised (Hazlett, 1985) that the chemicals given off by aggressing crayfish were not specific to that situation but rather were given off whenever an animal was disturbed. The following experiments test the idea that such chemicals are given off in a variety of situations and that conspecifics detecting these chemicals would respond in a similar manner irrespective of the source of disturbance to the emitting crayfish.



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If the above hypothesis is validated, it would complicate the testing of any chemical detection system since disturbed and undisturbed emitters of any pheromones would present different arrays of potential signals. Clearly it would be advantageous to be able to detect if others in the area (conspecific and heterospecific individuals) are stressed in some way.

## METHODS AND MATERIALS

The crayfish observed were hand-caught individuals of *Orconectes virilis* and male *O. rusticus* (no female *O. rusticus* were found at this time). The crayfish used as potential sources of chemicals were individuals of *O. virilis*, *O. rusticus*, and *O. propinquus*. The *Orconectes rusticus* were collected from a stream emptying from the southwest corner of Carp Lake near Paradise, Michigan. It should be noted that *O. rusticus* and *O. virilis* have overlapped in distribution only in recent times and are not believed to have cooccurred over evolutionary time (Capelli and Magnuson, 1983). The *O. propinquus* and most of the male *O. virilis* used were collected from the Maple River south of Pellston, Michigan. Most of the female *O. virilis* and a few males were from the north end of Burt Lake. All crayfish were sexually mature except one male *O. virilis* used as a source animal (see below). The tests were conducted in the Lakeside Laboratory of the University of Michigan Biological Station, Pellston, Michigan, during July and August 1984.

The basic method of data acquisition was similar to that outlined by Hazlett (1985). The postures of the crayfish recorded are associated with aggression/predator defense (raised postures), submission/resting (lowered postures), or general investigation of the habitat (neutral postures). For details see Hazlett (1985). Animals to be observed were placed in individual 10-gallon aquaria (50 × 26-cm bottom dimensions) which were visually isolated from each other. The water in each tank was 15 cm deep and was continually stongly aerated. Crayfish were allowed to acclimate to the observation aquaria for 48 hr prior to testing. Individuals of *O. rusticus* in particular continued to behave as if agitated (continual locomotion, frequent aggressive displays without known stimulation, climbing walls of aquaria) after only 24 hr acclimation, so a standard of two days was used for all animals to be observed or used as an undisturbed source animal. Cardboard blinds with a small viewing hole covered the sides of each aquarium.

Observations were always carried out between 1230 and 1700 hr. During a 10-min observation period, water was introduced into the aquarium in the corner farthest from the crayfish at a rate of 44 ml/min by a Masterflex brand peristaltic pump (model 7543-30, Cole Parmer) with a model 7015-20 head. The behavior of the test crayfish was categorized continuously into one of three positions (lowered, neutral, raised) for each of three body parts (cephalothorax or

body, chelipeds, and abdomen). The number of seconds during which each body part was in each position was recorded on an Esterline Angus chart recorder. The recorder and the pump were on a separate, movable table to minimize the possibility of vibrations from the equipment affecting the animals. Ten replicates of each test condition were run except for observations on female *O. virilis* ($N = 11$ for all three conditions) and the self-water condition (see below) for both *O. rusticus* males ($N = 9$) and *O. virilis* males ($N = 51$).

Each test animal was observed under three to four different test conditions, always including "self-water," but there was only one test per day per individual. The order of test conditions for any individual was random and at least three different conditions were tested on any day of observation. However, the testing of some conditions was completed earlier in the summer and others later, thus introducing the possibility of seasonal complications. Water introduced to an observation crayfish came from either the observed crayfish's own aquarium (self-water, control) or a visually isolated source aquarium containing 9 liters of water (20 cm deep) which was continually aerated and contained one or more crayfish. Several stones were used to fashion a shelter in the source aquarium and the resident was allowed 48 hr acclimation prior to use in any tests. In the self-water tests, both the input and output ends of the tubing were quietly introduced into the observed crayfish's aquarium and no observations were done if the crayfish showed any reaction to the introduction of the tubes.

Male *Orconectes rusticus* were observed during the introduction of three sources of water: (1) self-water, (2) an undisturbed, isolated male *O. rusticus* in the source aquarium, and (3) aggressing male *O. rusticus*. In condition 3, a second male *O. rusticus* was added to the source aquarium 15 sec prior to the start of observations of the test animal. The two males were of similar size and usually were very actively aggressing.

Female *O. virilis* were also observed during the introduction of three types of water: (1) self, (2) an undisturbed, isolated female *O. virilis* in the source aquarium, and (3) two aggressing female *O. virilis*.

In addition to self-water, the conditions under which male *O. virilis* were observed in the first set of tests (1-11) were as follows:
(1) undisturbed, isolated male *O. virilis;* (2) undisturbed, isolated female *O. virilis*, and (3) undisturbed, isolated male *O. rusticus*.

*Agonistically Stressed Crayfish:* These included (4) two aggressing male *O. virilis*; (5) two aggressing female *O. virilis*; (6) two aggressing male *O. rusticus*; and (7) two aggressing male *O. propinquus*.

Test conditions 1–7 were designed to see if all aggressing crayfish give off chemicals to which male *O. virilis* respond.

*Abiotic Stress:* These included (8) one heat-stressed male *O. virilis*. About 15 min prior to introducing source water, aquarium heaters started increasing the temperature of the source tank (initially 20–21 °C). When the temperature had increased by 8 °C, observations started and the elevated source aquarium

temperature was maintained during the observation period. The tubing carrying the source water was immersed in ice water and when the water was introduced to the observation aquarium it was elevated only 1.5–2.5°C and caused no measurable change in the temperature of the water in the observation aquarium. Heat was used as an easily controlled abiotic form of environmental stress which crayfish could encounter either naturally or as a result of human perturbations.

*Predator-Stressed Crayfish:* These included (9) one predator-stressed male *O. virilis*. The source aquarium was placed on the movable table holding the Esterline Angus recorder (both for easier access by the "predator" and to control for any possible substrate vibrations). The source crayfish was chased and prodded with an aquarium net by the observer during the observation period. Either the predator-defensive Aufbäumreflex or solid pinch on the net was elicited at least once every 30 sec.

(10) One predator-stressed male *O. propinquus* was subjected to conditions similar to nine, except there were no stones serving as a shelter in the source aquarium, thus allowing easier access by the "predator" to the source crayfish.

(11) Five predator-stressed male *O. propinquus* were subjected to the same conditions as in 10 except there were five individuals in the source aquarium and all were agitated.

In addition to the above tests, the responses of male *O. virilis* were recorded during the introduction of source aquarium water in two additional sets of tests.

*Aged Water tests:* (12) Forty-five minutes prior to the start of an observation period, one liter of water was scooped out of a source aquarium containing an undisturbed male *O. virilis*. This was set aside for 45 min and then used as the source water for introduction via the peristaltic pump. A given male *O. virilis* and its source aquarium was used only once per day for undisturbed male water.

(13) For 0-min *O. rusticus* aggressing males, two male *O. rusticus* were placed in a source aquarium and allowed to aggressively interact for 10 min. They were then removed and water from the now-empty aquarium used for introduction.

(14) For 15-min *O. rusticus* aggressing males the conditions were the same as 13 except that 15 min passed between removal of the *O. rusticus* and the start of observations.

(15) For 45-min *O. rusticus* aggressing males the conditions were the same as 13 and 14 except that 45 min passed between removal of the *O. rusticus* males and the start of observations.

Water from aggressing male *O. rusticus* was used in conditions 13–18 because of the strong responses of male *O. virilis* to aggressing male *O. rusticus* water and the lack of any response to undisturbed *O. rusticus* water, thereby eliminating any complication of responses to sex pheromones.

During observations, test animals usually showed no observable response to the introduction of water of any condition during the first several minutes.

Moreover, it seemed that once a test animal was behaving in a given manner, it persisted even after the introduction of water had ceased. To see if this was due to a threshold effect, the following tests were run:

(16) For 2-min active *O. rusticus* aggression, water from a source aquarium containing two aggessing male *O. rusticus* was introduced to an observation aquarium containing a male *O. virilis* for 2 min and the pump then turned off. Observation continued for 8 min without the introduction of any water.

(17) For 4-min active *O. rusticus* aggression conditions were the same as 16 except that 4 min of water introduction was followed by an additional 6 min of observation without water introduction.

(18) For 10-min active *O. rusticus* aggression conditions were the same as six.

All statistical analyses for differences in responses were by one-way ANOVAs of the number of seconds spent by crayfish in the various postures under the different test conditions. Since there was a set number of seconds (600) in an observation period and the three positions were exhaustive (the crayfish had to be in one of them), only two postures of each body part can be treated as independent. The neutral and raised postures were chosen for analysis as they were the best indicators of responsiveness in earlier tests (Hazlett, 1985), although the netural postures were infrequent in that study due to the test conditions utilized.

ANOVAs with pair-wise comparisons were run separately on the responses of female *O. virilis*, the responses of male *O. rusticus*, and the responses of male *O. virilis* to (a) conditions involving female *O. virilis* as a potential source, (b) all other conditions in the first set of tests, (c) aged water tests, and (d) threshold tests. Only those pair-wise comparisons that addressed biologically interesting questions were examined.

In just a few cases, the equality of variance assumption was not met by untransformed data, and so nonparametric analyses were also run wherever possible. In every case the Kruskal-Wallis scores were associated with probability values similar to those from the ANOVA analyses. Overall comparisons between treatments were either clearly insignificant ($P > 0.10$) or very significant ($<0.001$) by both tests. For ease of reading, only the ANOVA results will be mentioned.

## RESULTS

The time spent in the various postures by males of *Orconectes rusticus* during observation periods is shown in Table 1. There was no significant variation among treatments in any of the postures (overall $F$ values associated with $P > 0.10$).

Responses of female *Orconectes virilis* are shown in Table 2. Although the time spent in raised body positions appeared to be higher when undisturbed

TABLE 1. MEAN NUMBER OF SECONDS ($\pm$SD) SPENT IN VARIOUS POSTURES BY MALE *Orconectes rusticus* DURING 10-MIN OBSERVATION PERIODS WHILE DIFFERENT SOURCES OF WATER WERE INTRODUCED

| Source condition | Body neutral | Chelipeds neutral | Abdomen neutral | Body raised | Chelipeds raised | Abdomen extended |
|---|---|---|---|---|---|---|
| Self-water | 118 (124) | 70 (72) | 47 (47) | 120 (195) | 112 (196) | 109 (205) |
| Undisturbed male *O. rusticus* | 104 (121) | 114 (138) | 75 (111) | 152 (169) | 139 (171) | 117 (166) |
| Aggressing male *O. rusticus* | 111 (104) | 130 (131) | 68 (69) | 90 (135) | 82 (114) | 60 (120) |
| Probabilities associated with $F$ values | 0.96 | 0.54 | 0.74 | 0.79 | 0.73 | 0.71 |

TABLE 2. MEAN NUMBER OF SECONDS (±SD) SPENT IN VARIOUS POSTURES BY FEMALE *Orconectes virilis* DURING 10-MIN OBSERVATION PERIODS WHILE DIFFERENT SOURCES OF WATER WERE INTRODUCED

| Source condition | Body neutral | Chelipeds neutral | Abdomen neutral | Body raised | Chelipeds raised | Abdomen extended |
|---|---|---|---|---|---|---|
| Self-water | 157 (144) | 87 (79) | 57 (100) | 52 (95) | 45 (91) | 13 (32) |
| Undisturbed female *O. virilis* | 127 (99) | 97 (120) | 61 (91) | 113 (157) | 99 (48) | 74 (130) |
| Aggressing female *O. virilis* | 135 (128) | 83 (85) | 47 (65) | 55 (73) | 57 (67) | 14 (21) |
| Probabilities associated with *F* values | 0.84 | 0.92 | 0.92 | 0.37 | 0.47 | 0.13 |

conspecific water was introduced, there were no significant differences among treatments for any of the postures (overall *F* values associated with $P > 0.10$).

The responses of male *O. virilis* under all the conditions tested are shown in Table 3. Comparisons of the time spent in the various postures by treatment yielded very significant effects of treatment in every case (overall *F* values associated with $P < 0.0001$; between 21 and 29% of the variance explained by treatment effects).

The time spent in the various postures by male *O. virilis* did not differ among treatments when female *O. virilis* were in the source aquarium except for one comparison. When two females were interacting aggressively, more time was spent in the body neutral posture than during control periods ($P = 0.04$). All other comparisons were clearly insignificant. In the case of undisturbed, isolated male *O. rusticus* and two male *O. propinquus*, there were no significant differences in any of the postures when compared to control periods.

The time spent in postures by male *O. virilis* when undisturbed male conspecific (UMC) water was introduced was significantly different from control periods for only one posture (abdomen extended) (Table 4). However, two of those tests were inadvertently run with a source male *O. virilis* which was form II and probably sexually immature. If the responses of male *O. virilis* to only form I (sexually active form), undisturbed, male conspecifics are considered ($N = 8$), there are differences in the time spent in all three neutral postures compared to control periods. If the responses to UMC water aged 45 min are considered, clear differences appear, especially in the greater time spent in the raised postures (Table 4), compared to either control periods or the unaged UMC tests.

The responses of male *O. virilis* to water from aggressing male conspecifics was different from controls only in a greater time spent in the body neutral position ($P = 0.030$). Responses to water from one predator-stressed *O. propinquus* were different from controls in all three neutral positions ($P = 0.029$, 0.014, and 0.020 for body, cheliped, and abdomen).

Four other test conditions (predator-stressed male *O. virilis*, five predator-stressed male *O. propinquus*, aggressing male *O. rusticus*, and heat-stressed male *O. virilis*) were very similar in the responses elicited. For all four conditions, (a) all three neutral positions were very significantly different from control periods (all $P < 0.001$), (b) in almost all there were no differences from control periods in the time spent in the raised positions ($P > 0.10$, except for aggressing *O. rusticus* which did elicit an increase in raised postures compared to control periods), and (c) very few differences among the responses shown under these four test conditions. The latter is shown in Table 5, in that among the 36 pairwise comparisons possible, the only differences were greater raised time with aggressing *O. rusticus* than with predator-stressed conditions and less time in neutral postures with heat-stressed compared to predator-stressed *O. virilis*. The

differences among these four conditions are not large compared to the differences between each of the four and the control period.

The responses of male *O. virilis* to aged water in which male *O. rusticus* had been aggressing did not differ from the responses shown to unaged water (containing aggressing *O. rusticus*). The overall $F$ values comparing the four treatments (unaged, aged 0 min, 15 min, and aged 45 min *O. rusticus* aggressing males) were all associated with $P > 0.10$, except the body raised posture (overall $P = 0.0853$) for which the 15- and 45-min aged water was different from the 0-aged water ($P = 0.036$ and $P = 0.046$).

The response of male *O. virilis* in the threshold tests showed significant variation among treatments ($P < 0.01$) for all six postures, especially in the time spent in neutral postures ($P < 0.0006$). Two minutes of aggressing *O. rusticus* water did not elicit responses different from control periods (all postures $P > 0.10$), while both 4 and 10 min of active aggressing *O. rusticus* elicited significantly more time in all three neutral positions compared to controls ($P < 0.001$) but did not differ from one another ($P > 0.10$).

## DISCUSSION

Male *Orconectes virilis* reacted to waters in which other male crayfish were stressed, whether those crayfish were conspecifics or members of other species. The responses were similar in all cases and consisted of increased time spent in "neutral" postures during the observation periods.

The "neutral" label could be replaced by "intermediate," since the postures were physically between the resting/lowered postures and the aggressive/raised postures. Normally, as during the control (self-water) periods, individuals of most crayfish are relatively inactive during the midafternoon hours and are in the lowered postures unless disturbed. If the source of disturbance is strong and direct (e.g., predator attack, aggressing conspecific), the crayfish responds with raised postures. As proposed earlier (Hazlett, 1985), the intermediate (neutral) postures seem to reflect a change in alertness associated with a low level of disturbance of a less well-defined nature. The positions of the cephalothorax, chelipeds, and abdomen seem linked with an investigatory or wary mood. It would appear that male *O. virilis* can chemically detect if other male crayfish in the area have been stressed or disturbed by something and are then more attentive to stimuli in general. This response would increase the recepient's chances of detecting the source of disturbance and perhaps avoiding stressful situations.

The lack of a significantly different response by male *O. virilis* to aggressing female *O. virilis* may well result from the complications of sex phermone detection. As reported earlier (Hazlett, 1985), male *O. virilis* can chemically

TABLE 3. MEAN NUMBER OF SECONDS ($\pm$SD) SPENT BY MALE *Orconectes virilis* IN VARIOUS POSTURES DURING 10-MIN OBSERVATION PERIODS WHILE WATER FROM VARIOUS SOURCES WAS INTRODUCED

| Source condition | Body neutral | Chelipeds neutral | Abdomen neutral | Body raised | Chelipeds raised | Abdomen extended |
|---|---|---|---|---|---|---|
| Self-water | 39 (70) | 21 (54) | 19 (45) | 4 (21) | 2 (10) | 2 (8) |
| Undisturbed male *O. virilis* | 102 (107) | 103 (114) | 74 (103) | 42 (53) | 35 (39) | 35 (51) |
| Aggressing male *O. virilis* | 144 (144) | 91 (95) | 51 (93) | 34 (98) | 31 (99) | 28 (91) |
| Undisturbed female *O. virilis* | 82 (103) | 54 (103) | 26 (55) | 9 (15) | 6 (13) | 1 (3) |
| Aggressing female *O. virilis* | 139 (173) | 85 (112) | 61 (113) | 19 (36) | 22 (39) | 5 (10) |
| Undisturbed male *O. rusticus* | 66 (107) | 61 (107) | 57 (121) | 18 (39) | 20 (43) | 9 (22) |
| Aggressing male *O. rusticus* | 223 (179) | 241 (191) | 166 (192) | 67 (118) | 58 (117) | 39 (92) |
| Aggressing male *O. propinquus* | 98 (144) | 77 (115) | 76 (125) | 18 (34) | 17 (34) | 10 (26) |
| Heat-stressed male *O. virilis* | 190 (153) | 124 (88) | 82 (109) | 26 (76) | 26 (79) | 25 (81) |
| Predator-stressed male *O. virilis* | 322 (203) | 240 (195) | 251 (218) | 7 (14) | 3 (6) | 1 (3) |
| Predator-stressed male *O. propinquus* | 147 (184) | 126 (165) | 123 (196) | 18 (47) | 20 (38) | 1 (3) |
| Predator-stressed five male *O. propinquus* | 269 (212) | 182 (173) | 169 (160) | 10 (20) | 15 (23) | 1 (1) |

| | | | | | | |
|---|---|---|---|---|---|---|
| 45-min aged water undisturbed male | | | | | | |
| *O. virilis* | 167 (99) | 147 (97) | 148 (116) | 183 (168) | 144 (143) | 139 (128) |
| Probabilities | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| Aged water tests | | | | | | |
| 0-min aged water aggressing | | | | | | |
| *O. rusticus* | 349 (119) | 307 (145) | 208 (180) | 51 (56) | 43 (56) | 35 (47) |
| 15-min aged water aggressing | | | | | | |
| *O. rusticus* | 181 (208) | 131 (166) | 141 (204) | 3 (8) | 3 (7) | 1 (1) |
| 45-min aged water aggressing | | | | | | |
| *O. rusticus* | 260 (210) | 185 (157) | 201 (187) | 6 (14) | 11 (27) | 5 (12) |
| Probabilities | 0.227 | 0.120 | 0.404 | 0.085 | 0.209 | 0.230 |
| Threshold Effects Tests | | | | | | |
| 2-min active aggressing | | | | | | |
| *O. rusticus* | 102 (165) | 54 (104) | 58 (130) | 0 | 0 | 0 |
| 4-min active aggressing | | | | | | |
| *O. rusticus* | 228 (158) | 143 (141) | 126 (138) | 12 (24) | 12 (30) | 0 |
| 10-min active aggressing | | | | | | |
| *O. rusticus* | 180 (166) | 139 (137) | 136 (187) | 49 (109) | 41 (90) | 28 (83) |
| Probabilities | <0.001 | <0.001 | <0.001 | 0.032 | 0.018 | 0.075 |

TABLE 4. PROBABILITIES ASSOCIATED WITH $F$ VALUES FROM ANOVA PAIR-WISE COMPARISONS FOR RESPONSES OF MALE *O. virilis* TO UNDISTURBED MALE *O. virilis* WATER (AGED AND UNAGED) AND CONTROL PERIODS

| Comparison | Body neutral | Chelipeds neutral | Abdomen neutral | Body raised | Chelipeds raised | Abdomen extended |
|---|---|---|---|---|---|---|
| Self vs. all UMC unaged | 0.201 | 0.055 | 0.229 | 0.070 | 0.086 | 0.044 |
| Self vs. form I UMC unaged | 0.011 | 0.000 | 0.005 | 0.081 | 0.079 | 0.058 |
| Self vs. aged UMC | 0.009 | 0.003 | 0.005 | 0.000 | 0.000 | 0.000 |
| Unaged vs. aged UMC | 0.305 | 0.419 | 0.215 | 0.000 | 0.000 | 0.000 |

TABLE 5. PROBABILITIES ASSOCIATED WITH $F$ VALUES FROM ANOVA PAIR-WISE COMPARISONS OF TIME SPENT IN VARIOUS POSTURES BY MALE *O. virilis* UNDER FOUR DIFFERENT TEST CONDITIONS

| Comparison | Body neutral | Chelipeds neutral | Abdomen neutral | Body raised | Chelipeds raised | Abdomen extended |
|---|---|---|---|---|---|---|
| Aggressing *O. rusticus* | | | | | | |
| vs. predator-stressed *O. virilis* | 0.124 | 0.992 | 0.154 | 0.024 | 0.027 | 0.075 |
| vs. predator-stressed *O. propinquus* (5) | 0.475 | 0.286 | 0.958 | 0.032 | 0.083 | 0.070 |
| vs. heat-stressed *O. virilis* | 0.605 | 0.034 | 0.155 | 0.120 | 0.192 | 0.539 |
| Predator-stressed *O. virilis* | | | | | | |
| vs. predator-stressed *O. propinquus* (5) | 0.407 | 0.291 | 0.170 | 0.907 | 0.626 | 0.973 |
| vs. heat-stressed *O. virilis* | 0.040 | 0.035 | 0.004 | 0.478 | 0.359 | 0.243 |
| Heat-stressed *O. virilis* | | | | | | |
| vs. predator-stressed *O. propinquus* (5) | 0.219 | 0.289 | 0.140 | 0.552 | 0.666 | 0.230 |

distinguish between male and female conspecifics, and the response to undisturbed females is to assure submissive postures, the latter being part of courtship behavior (Ameyaw-Akumfi, 1979). If males' responses to sex pheromones are stronger than responses to disturbance pheromones, demonstration of release of the latter by females would be complicated.

The lack of a response by male *O. virilis* to water containing two male *O. propinquus* was clearly related to the low level of (aggressive) stress of the *O. propinquus*. In every replicate, the two *O. propinquus* spent the majority of the test period quietly resting in different parts of the source aquarium, not interacting, whereas when two male *O. virilis* or *O. rusticus* were placed in the source tank they interacted aggressively the vast majority of the testing period. Thus this situation actually tested for responses to apparently undisturbed male *O. propinquus*, and there were no differences between that condition and control periods. Male *O. virilis* showed no observable responses to water containing undisturbed male nonconspecific crayfish (*O. propinquus* and *O. rusticus*), a result similar to that reported for (*O. rusticus*) by Tierney and Dunham (1984).

The responses of male *O. virilis* to water containing one predator-stressed *O. propinquus* just were significantly different from control periods. However, water from five predator-stressed *O. propinquus* did elicit a clear response, thus male *O. virilis* do react to chemicals from stressed individuals of that species. The weak response to the single stressed *O. propinquus* condition is probably related to the size of *O. propinquus* used as a potential source. This is a smaller species in general, and the mean cephalothorax length of the source individuals of *O. propinquus* was 26 mm compared to 41 mm, 40 mm, and 44 mm for male *O. virilis*, female *O. virilis*, and *O. rusticus*, respectively. In the test situation used, one crayfish that size may not have been able to produce enough of the purported chemical(s) to pass the detection threshold of the system in many of the replicates.

The lack of female *O. virilis* response differences to waters of different conditions is perplexing but consistent with the results from earlier tests (Hazlett, 1985). Female *O. virilis* do respond differently to waters containing conspecific vs. heterospecific males (Tierney and Dunham, 1984), but they showed no response differential to waters of differing conditions in the present study. Additional types of tests are needed such as those of Rose and Casper (1980) which did demonstrate a number of chemically mediated responses in female *Procambarus clarkii*.

The clear lack of differences in the responses of male *O. rusticus* may have been an artifact of the testing situation. Individuals of *O. rusticus* were very easily disturbed in the laboratory situation and did not appear fully acclimated to the aquaria (observation or source) even after two days. The individual *O. rusticus* observed may have been unacclimated and thus no differential in responses detectable (a "wary" crayfish cannot become "wary"). In addition, the

source water (self, isolated, and aggressing) may all have been from partially disturbed crayfish, thus the lack of a differential in responses to such waters.

As in any study, the presence or absence of differences in responses to various conditions is, of course, limited by the behavioral data taken. The nine categories used (three body parts, three positions) were chosen for their utility in recognizing responses to sex pheromones (Ameyaw-Akumfi and Hazlett, 1975; Hazlett, 1985). Obviously in every case where no response differential was detected among or between treatments, it may have been that the appropriate behavior patterns were not recorded (see Rose, 1982). Thus while the data taken indicate differentials between control and many test situations, the lack of differences among various stressed situations could be due to the limited number of patterns measured (Ameyaw-Akumfi and Hazlett, 1975; Christofferson, 1978; Gleeson, 1980). The detection of disturbance pheromones requires undisturbed animals for control periods. The lack of such controls may well lead to problems of data interpretation [see the sex pheromone discussion of Hazlett (1984), Thorp (1984), and Rose (1984)].

The responses of male *O. virilis* to undisturbed male conspecifics (UMC) were similar to that reported for this species (Hazlett, 1985), when only form I males were tested. The results of the two replicates which involved a form II source male can only be taken as suggestive that form II males do not give off a sex pheromone. It is clear, however, that even considering only the responses for form I UMC, the response level was higher in the aged UMC water tests compared to the unaged water. It is unlikely that the potency of the sex pheromone increases upon aging 45 min [although the chemical(s) involved do remain active at least that long]. A more probable explanation is the difference in time of testing. Most of the unaged UMC tests were run in June when many males had just recently molted to the sexually active form I. The aged UMC tests were conducted in August when some sexual activity by *O. vivilis* was seen in the field and laboratory. Seasonal variation in behavior has been reported for most temperate zone crayfish (e.g., Thorp, 1978).

Whatever the chemical nature of the disturbance pheromone, it seems somewhat stable. Although there seemed to be a trend towards reduced effectiveness of aggression-stressed water after 45–55 min of standing at room temperature, this was not significant.

The tests with 2, 4, and 10 min of aggressing *O. rusticus* water introduction indicated a threshold effect in two ways. The clear lack of responses to just 2 min of water introduction suggests that there is a response threshold—88 ml of water was insufficient to elicit a response while 176 ml was sufficient. This could be due to a detection threshold (insufficient concentration of molecules for sensory detection given the pattern water movement) and/or a motivational threshold. The latter refers to central nervous system influences which determine the behavior state or drive level of an animal. The fact that 176 ml of water elicited

the same responses as 440 ml of water (10 min of introduction) points toward a motivational threshold mechanism. Once sufficient information about disturbances is detected, the animal is wary for some minutes even if added input is not forthcoming. The results of Rose and Casper (1980) with female *Procambarus clarkii* also indicated a threshold effect.

The results of this study take care of several problems with an earlier study of responses to pheromones by individuals of *Orconectes virilis* (Hazlett, 1985). First, the aggressing crayfish condition has two animals in the source aquarium rather than one. However, similar responses to waters were obtained in this study with one *O. virilis* when it is stressed (heat or predator). Secondly, the fact that self-water tests involved water from an aquarium without a rock shelter while the source tanks had rock shelters raises a problem of confounded differences in conditions. Yet the predator-stressed *O. propinquus* tests were run without any rock shelters in the source aquarium, and those tests yielded results similar to other stressed-crayfish conditions. In addition, five tests (not included in the Results section) were run with male *O. virilis* as observed crayfish in which I thought there was a male *O. virilis* under the rocks in the source aquarium. After observations were completed, I discovered the source tank was empty. In those five tests, the crayfish were in fully lowered postures for the entire 10 min, thus these served as a control (inadvertantly) both for source aquarium features unrelated to the crayfish present and for possible observer bias.

While these tests were obviously limited taxonomically to crayfish, it seems reasonable to expect disturbance semiochemicals in many species, especially aquatic organisms. Animal metabolisms are likely to shift slightly in some way (qualitatively or quantitively) when disturbed, and it would be to any individual's advantage to detect disturbances in their environment and learn to respond appropriately (Valenta and Rigby, 1968).

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# ISOFLAVONOID FEEDING DETERRENTS FOR *Costelytra zealandica*
## Structure–Activity Relationships

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**Abstract**—A number of naturally occurring isoflavonoids of differing substitution patterns and oxidation states have been tested for feeding deterrent activity in a bioassay with larvae of *Costelytra zealandica* White. The most active deterrents, which reduced feeding significantly at 0.2–1.0 μg/g, are those compounds containing a ring B-fused cycloprenoid moiety. The least active compounds were highly oxidized coumestans and isoflavones. The ring B-fused cyclic isoprenoid moiety and the presence of a 2′-oxy function appear to be structural features important for high activity. It is suggested that the feeding deterrent activity of isoflavonoids relates to their stereochemistry and that the most active compounds have or can adopt a similar nonplanar molecular shape with a similar arrangement of polar and lipophilic groups.

**Key Words**—Isoflavonoids, structure–activity relationships, insect feeding-deterrent activity, *Costelytra zealandica*, Coleoptera, Scarabaeidae, stereochemistry.

## INTRODUCTION

Recent work has shown that the subterranean, root-feeding larvae of the beetle *Costelytra zealandica* White (Coleoptera: Scarabaeidae), which inflict major damage on New Zealand pastures, are deterred from feeding by several isoflavonoid constituents of legume roots (Russell et al.,1978; Sutherland et. al., 1980; Lane et. al., 1985). Several of the most active compounds have also been recognized as phytoalexins (antifungal metabolites elicited in response to stress



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or infection) in the aerial parts of legumes. This observation led to the proposal that in the defense strategy of these plants, the isoflavonoids may serve two different and perhaps independent roles, as phytoalexins and also as insect feeding deterrents (Sutherland et al., 1980). Isoflavonoids are only one of several classes of compounds implicated in legume resistance to grass grub (Sutherland et al., 1975, 1982a, b; Hutchins et al., 1984).

The isoflavonoids present in different legumes differ in structural type, yield, and level of feeding deterrent activity. In order to understand and predict the effect of the isoflavonoids of a particular legume on the feeding of *C. zealandica* larvae, it is important to determine whether deterrent activity can be related quantitatively to defined structural features of the constitutive isoflavonoid molecules. It is generally accepted that molecular size and shape are important determinants for semiochemical activity (Silverstein, 1979), and studies with insect feeding deterrents have shown that redox potential, intramolecular hydrogen bonding capability (Norris, 1977), and functional group stereochemistry (Kubo and Ganjian, 1981; Kojima and Kato, 1981) can be important factors in relating molecular structure to insect gustatory response.

In this study we have examined the effect of a range of naturally occurring isoflavonoids, some synthetic analogs, and some simple chromenes on the feeding behavior of *C. zealandica* larvae in order to determine those molecular features that are important for high feeding-deterrent activity. We have drawn together data from our earlier studies on isoflavonoids (Sutherland et al., 1980; Lane et al., 1985) and new data acquired for this study, and analyzed them with a uniform statistical treatment.

## METHODS AND MATERIALS

*Feeding Deterrent Assay.* Field-collected third-instar larvae of *Costelytra zealandica*, which had been starved for 24 hr, were enclosed individually in 5.2-cm glass Petri dishes with a 4% agar–4% cellulose powder disk (1.5 cm). Feeding-deterrent activity was assessed by comparing 24-hr fecal pellet counts of larvae offered disks containing both standard feeding stimulant (0.1 M sucrose + 0.01 M ascorbic acid) (Sutherland, 1971) and test material with similar counts of larvae offered disks containing feeding stimulants only (control) and of a third group offered disks prepared with neither (blank). Twenty larvae were tested with each medium. Blank and control treatments were run on all feeding occasions, and the difference between them was taken as the standard stimulated response (standard).

Most isoflavonoids were tested at four concentrations in the disk, usually 2, 20, 100, and 200 $\mu$g/g. Lack of material limited the range of some compounds to three concentrations, while the activity of a few others required a wider test range. The required amount of each compound was dissolved in solvent, added

to a weighed sample of cellulose powder, and the solvent evaporated. Test media were then prepared by adding an appropriate amount of hot 4% agar solution to each cellulose powder sample, stirring vigorously, and pouring a measured amount into a glass Petri dish lid containing either sufficient concentrated sucrose and ascorbic acid solution to make up the desired final concentration (test and control) or an equivalent volume of distilled water (blank). The combined ingredients were then stirred thoroughly and set aside to cool. This procedure was adopted to minimize oxidation of the ascorbic acid. Test disks were then cut from the media with a cork borer.

*Statistical Treatment of Data.* Although the fecal pellet count is the most accurate, reproducible, and convenient method of measuring ingestion by *C. zealandica* larvae (Sutherland, 1971), the feeding behavior of individual insects presented with the same medium at the same time often differs widely (for example, see Figure 1). Precise quantification of the behavior and comparison of response to often quite similar test chemicals was therefore difficult. Instead of the mean fecal pellet count, a Hodges-Lehmann estimate (Hollander and Wolfe, 1973) was used as the measure of the overall level of response of all insects tested at a particular concentration of a test compound. As used here, it is the median of all possible pairwise averages of individual fecal pellet counts. Its advantage is that it is unaffected by a few very large or very small counts, which were a feature of some sets of the results.

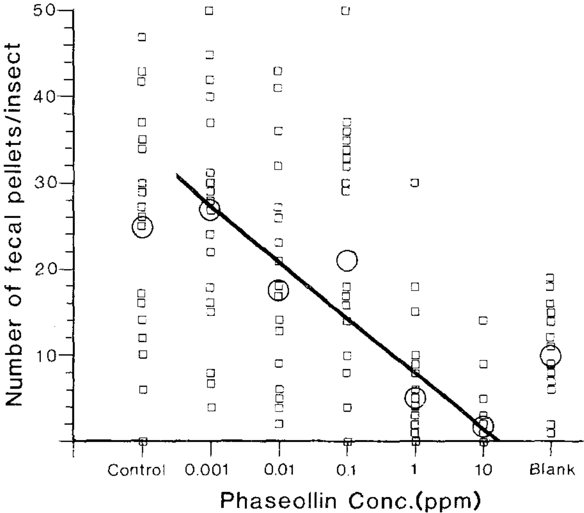


FIG. 1. Result of one assay of the feeding response of 20 third-instar *Costelytra zealandica* larvae to each of five concentrations of phaseollin and to control and blank treatments. □ = individual fecal pellet counts; ○ = Hodges-Lehmann estimate; line shows estimated dose–response curve.

The data were analyzed by a procedure analogous to probit analysis. The Hodges-Lehmann estimates were plotted against log concentration. A straight line was fitted to the points obtained and the $FD_{50}$ was calculated as the concentration corresponding to the midpoint between the blank and the control fecal pellet counts, i.e., that concentration at which stimulated feeding was reduced to 50% of the standard. The slope of each line was also calculated. Figure 1 shows individual pellet counts, Hodges-Lehmann estimates, and the fitted line for one of the trials with phaseollin (I). Providing a suitable concentration range was used, the assumption of linear change in feeding response with log-dose appeared adequate; the data did not allow the fitting of more complex models such as a sigmoid curve.

In a few cases, all concentrations tested depressed feeding to a level close to or even below that for the blank. It was then clear that the $FD_{50}$ was less than the lowest concentration tested. These experiments were repeated with a more suitable range of concentrations.

In order to calculate standard errors for the $FD_{50}$ values and the slopes, pooled standard errors of the relevant Hodges-Lehmann estimates were required. For each Hodges-Lehmann estimate, the standard error was estimated at the $100\ (1 - \alpha)\,\%$ confidence level by the method of Hollander and Wolfe (1973). The term $\alpha$ was set at 0.32 to give a confidence interval of twice the standard error; smaller values of $\alpha$ make the interval (and hence the standard error) sensitive to extreme data values.

For each experiment, the root mean square of the standard errors of the Hodges-Lehmann estimates of responses at all concentrations of the test compound was calculated as an overall (pooled) estimate of the error. This allowed calculation of standard errors for the slope and intercept of the regression line. Following Finney (1971, p. 78) these lead to standard error limits $\log FD_{50} - d$, $\log FD_{50} + d$, where standard errors of blank and control are also incorporated in $d$. For the $FD_{50}$, the limits are ($FD_{50} \div c$, $FD_{50} \times c$), where $c$ = antilog ($d$) (Tables 1–4). In order to test whether the slope of the regression line differed from zero, it was divided by its standard error and the absolute value taken, to give a statistic $z$. A value of $z$ greater than 1.65 indicated statistical significance, corresponding to the one-sided 5% point for the normal distribution. Where a compound was tested twice, the requirement was at least one of the two $z$ values should be more than 1.95; this gives an overall test with a nominal significance level of 5%. For three tests, the requirement was similarly increased.

*Chemicals.* The following compounds were obtained commercially: VIII (Aldrich) XV and XVII (ICN Pharmaceuticals Inc.), XXVII (Eastman, Kodak Ltd.), XXXIV and XXXV (Calbiochem). L. Jurd, USDA, Albany, CA, provided compounds XXVIII–XXXI and O. Gottlieb, Instituto de Quimica, Universidade de Sao Paulo, Brazil, provided compounds XI and XXXII. The source

I. R=H
II. R=Me

III.

IV. R=H
V. R=Me

VI.

VII.

VIII.

IX. R'=H
X. R'=Me

XI.

of compounds I, IV, V, IX, X, XII–XIV and XVIII has been previously described (Sutherland et al., 1980). The isolation of VI, VII, XVI, and XX–XXIII as insect feeding deterrents and antifungal components from the roots of *Lupinus angustifolius* is the subject of a separate report (Lane et al., 1985). The following compounds were isolated or synthesized as described.

*7-O-Methylphaseollin* (*II*). Phaseollin (I) was treated with ethereal diazomethane in MeOH. Crystallization from aq. MeOH gave II, mp 120–122°C. Comparison of the UV, mass spectral, and [$^{1}$H]NMR data with those reported for phaseollin (I) (Perrin et al., 1972) indicated the structure II. UV (EtOH)

$\lambda_{max}$ 207 ($\epsilon$ 47,000), 230.5 ($\epsilon$ 37,000), 279.5 ($\epsilon$ 13,000), 285 sh ($\epsilon$ 11,000), 315.5 nm ($\epsilon$ 3,000); EIMS (probe, 70 eV) *m/z* (relative intensity) 336 ($M^+$, 63), calcd 336.13615, found 336.13699, 322 (24), 321 (100), 293 (40), 160 (26); [$^1$H]NMR ($CDCl_3$, 80 MHz) $\delta$ 7.43 (1 H, d, $J$ = 8.5 Hz, H-5), 6.93 (1 H, d, $J$ = 8.1 Hz, H-6′), 6.62 (1 H, dd, $J$ = 2.5, 8.5 Hz, H-6), 6.49 (1 H, d, $J$ = 9.9 Hz, H-$\alpha$), 6.45 (1 H, d, $J$ = 2.6 Hz, H-8), 6.32 (1 H, dd, $J$ = 0.7, 8.0 Hz, H-5′), 5.55 (1 H, d, $J$ = 9.9 Hz, H-$\beta$), 5.48 (1 H, br d, $J$ = 6.3 Hz, H-4), 4.2 (1 H, m, H-2), 3.77 (3 H, s, $OCH_3$), 3.6–3.4 (2 H, m, H-2, H-3), 1.41 (3 H, s, $CH_3$), 1.38 (3 H, s, $CH_3$). (Numbering as for structure I).

*Phaseollidin (III) and Kievitone (XXIV).* Etiolated hypocotyls of *Phaseolus vulgaris* cv. Tendergreen were treated with conidial suspensions of *Sclerotinia fructicola* (Wint.) Rehm, Saccardo in 0.5 ppm cycloheximide and incubated 24 hr at 25°C. Ethanolic extracts of freeze-dried hypocotyls were chromatographed on silica gel with $CHCl_3$–EtOH and petroleum ether–$Et_2O$.

Phaseollidin (III), purified by repeated chromatography on Sephadex LH-20 with $CHCl_3$ and $CHCl_3$–EtOH (19 : 1) gave UV, EIMS, and [$^1$H]NMR spectra in agreement with reported data (Perrin et al., 1972).

Kievitone (XXIV) was purified by repeated chromatography on Sephadex LH-20 with $CHCl_3$–petroleum ether–EtOH (10 : 10 : 5). The UV, EIMS, and [$^1$H]NMR were in agreement with data reported by Smith et al. (1973).

*2′-Hydroxyformononetin (XIX).* This was synthesized from 2,4-dihydroxyacetophenone and 2-hydroxy-4-methyoxybenzaldehyde as described by Farkas et al. (1974). The mp 220–222°C (lit. 220–221°C, Farkas et al., 1974), UV, and [$^1$H]NMR data were in agreement with reported values (Dewick, 1977; Braz Filho et al., 1977).

*(±)-Medicarpin (Racemic XII) and (±)-Vestitol (Racemic IX).* Catalytic hydrogenation of XIX in HOAc at room temperature and atmospheric pressure over 10% Pd/carbon and column chromatography on silica gel with petroleum ether–$Et_2O$ afforded (±)-medicarpin (racemic XII), mp 198–200°C (lit. 194–195°C, Cocker et al., 1965), and (±)-vestitol (racemic IX), mp 173–175°C (lit. 173–175°C, Farkas et al., 1974). The UV, MS, and [$^1$H]NMR spectra were identical to those for (−)-medicarpin (XII) and (−)-vestitol (IX) respectively.

*(±)-Methylequol (XXV).* Catalytic hydrogenation of XVIII in HOAc over 10% Pd/carbon (Nottle and Beck, 1974) gave XXV, mp 160–161°C (lit. 160°C, Nottle and Beck, 1974). The MS and [$^1$H]NMR data were in accord with those reported by Breytenbach et al. (1981).

*Dihydroformononetin (XXVI).* Catalytic hydrogenation of the acetate of XVIII in acetone at room temperature and atmospheric pressure over 10% Pd/carbon and mild hydrolysis with $NaHCO_3$–MeOH gave XXVI, mp 197–198°C (lit. 197–199°C, Breytenbach et al., 1981). The MS and [$^1$H]NMR data were in agreement with those reported by Breytenbach et al. (1981).

*Epirotenone (XXXIII).* Rotenone (VIII) was converted to the crystalline

XII.

XIII. R'=R''=H
XIV. R'=Me, R''=OH

XV. R'=R''=H
XVI. R'=OH, R''=H
XVII. R'=H, R''=Me

XVIII. R=H
XIX. R=OH

XX. R=H
XXI. R=OH

XXII.

XXIII.

XXIV.

epimeric mixture "mutarotenone" by treatment with NaOAc in EtOH as described by Crombie et al. (1961). The mixture was separated by preparative recycle HPLC on a silica gel column with petroleum ether–EtOAc (5 : 2) using a Waters Prep-500A chromatograph, with refractive index detection. The separation was monitored by analytical HPLC on a 25-cm, 10$\mu$m silica gel column with hexane–EtOAc (5 : 1) with UV detection at 280 nm, and rotenone could not be detected in the purified fraction ($<1\%$). Crystallization from MeOH gave XXXIII mp 88–91 °C, $[\alpha]_D + 60°$ (c 1.1, benzene) [lit. mp 90°C, $[\alpha]_D + 75.6°$ (c 0.6), Crombie et al., 1961]. The [$^{13}$C]NMR ($CDCl_3$, 20 MHz) data for

XXV. R=$H_2$
XXVI. R=O

XXVII. R'=OH, R''=H
XXVIII. R'=H, R''=OH

XXIX. R=H
XXX. R=OH

XXXI.

XXXII.

XXXIII.

XXXIV. R=H
XXXV. R=OMe

XXXVI.

XXXVII.

XXXIII are closely similar to those for rotenone (VIII) recorded under identical conditions: epirotenone (XXXIII), $\delta$ 188.9 (C-4), 167.3 (C-7), 157.9 (C-9), 149.5 (C-4'), 147.4 (C-2'), 143.9 (C-5'), 143.0 (-C=), 129.9 (C-5), 113.4 (C-10), 112.9 (C-8), 112.4 (=$CH_2$), 110.5 (C-6'), 104.9 (C-6), 100.9 (C-3'), 87.9 (furanoid CH-O), 72.2 (C-2), 66.3 ($CH_2$-O), 56.3 ($OCH_3$), 55.8 ($OCH_3$), 44.5 (C-3), 31.1 (Ar-$CH_2$), 17.1 (-$CH_3$); rotenone (VIII), $\delta$ 188.8, 167.3, 157.8, 149.5, 147.3, 143.8, 143.0, 129.8, 113.2, 112.9, 112.4, 110.5, 104.8, 100.9, 87.7, 72.2, 66.2, 56.3, 55.7, 44.5, 31.2, 17.0. The assignments for XXXIII are based on those reported by Crombie et al. (1975) for VIII, and the numbering used is that shown for I and VIII. The assignments are supported by multiplicities determined using a DEPT pulse sequence (Doddrell et al., 1982).

The [$^1$H]NMR ($CDCl_3$, 80 MHz) data for XXXIII are also very similar to those reported for VIII (Carlson et al., 1973). $\delta$ 7.81 (1 H, d, $J$ = 8.5 Hz, 5-H), 6.77 (1 H, d, $J$ = 0.6 Hz, 6'-H), 6.47 (1 H, d, $J$= 8.6 Hz, 6-H), 6.42 (1 H, s, 3'-H), 5.26 (1 H, br t, $J$ = 8.9 Hz, furanoid CH-O), 5.01 (1 H, br s,

C=CH), 4.9 (2 H, m, C=CH, 2-H), 4.59 (1 H, dd, $J$ = 3.0, 11.9 Hz, 2a-H), 4.14 (1 H, br d, $J$ = 11.6 Hz, 2a-H), 3.81 (1 H, br d, $J$ = 4.1 Hz, 3-H), 3.77 (3 H, s, $OCH_3$), 3.74 (3 H, s, $OCH_3$), 3.29 (1 H, dd, $J$ = 15.8, 9.6 Hz, Ar-CH), 2.92 (1 H, dd, $J$ = 15.9, 8.3 Hz, Ar-CH), 1.70 (3 H, s, $CH_3$). The observed couplings are almost identical to those reported for VIII by Carlson et al. (1973), and any chemical shift differences are slight. The coupling of 4.1 Hz for 3-H, obscured by the $OCH_3$ signals in $CDCl_3$, was determined from a spectrum in pyridine-$d_5$ (Carlson et al., 1973). Homonuclear shift-correlated two-dimensional NMR experiments (COSY; Bax and Freeman, 1981) showed an identical pattern of connectivities for XXXIII and VIII, and closely similar resolved subspectra for the protons in the furanoid ring and those about the C/D ring junction. These data indicate the heterocyclic ring conformations of XXXIII and VIII are closely similar, despite the different relative configurations in the two compounds.

*Dihydroprecocene II (XXXVI)*. Catalytic hydrogenation of XXXV in HOAc at room temperature and atmospheric pressure over 10% Pd/carbon gave XXXVI, mp 62°C [lit 61–62°C, Ahluwalia et al. (1982)] with [$^1$H]NMR data in agreement with that of Ahluwalia et al. (1982).

*6-Acetyl-5-hydroxy-2,2-dimethylchromene (XXXVII)*. This was prepared from 2,4-dihydroxyacetophenone and 3-hydroxy-3-methylbutanal dimethyl acetal in pyridine as described by Bandaranayake et al. (1971). The mp and [$^1$H]NMR data were in agreement with those reported by Bandaranayake et al. (1971).

## RESULTS

Table 1 gives $FD_{50}$ values and slopes of dose–response lines, together with the standard errors, for compounds giving a significant correlation between dose and feeding response. The criteria for inclusion in Table 1 are that on at least one occasion: (1) the dose–response gave an $FD_{50}$ not more than 1000 $\mu$g/g (five times the maximum concentration tested), and (2) the slope of the regression line was negative and significantly different from zero at the 5% level. Different sets of values for a compound correspond to measurements on different days.

The active feeding deterrents in Table 1 are listed in decreasing order of activity (as measured by the $FD_{50}$), although the wide error limits of the $FD_{50}$ values imply that the ranking is approximate. No account has been taken of differences in the slopes of the dose–response lines. While these isoflavonoids, as a group, all show feeding-deterrent activity, the table spans a very wide range of activities. With $FD_{50}$ values of 0.02 $\mu$g/g and 0.03 $\mu$g/g, phaseollinisoflavan (IV) and phaseollin (I) are among the most active insect feeding deterrents yet recorded. The variation between the $FD_{50}$ values of compounds tested on dif-

TABLE 1. EFFECT OF ISOFLAVONOIDS ON FEEDING OF *Costelytra zealandica* LARVAE: SIGNIFICANT DOSE-RESPONSE CORRELATION

| Compound | Feeding response | | |
|---|---|---|---|
| | $FD_{50}$ ($\mu$g/g) | $c$ (×, ÷)[a] | Slope of dose-response line |
| (−)-Phaseollinisoflavan (IV) | 0.02 | 4.7 | −3.8 |
| (−)-Phaseollin (I) | 0.03 | 2.2 | −6.3 |
| (−)-Rotenone (VIII) | 0.06 | 3.3 | −4.3 |
| (+)-2′-*O*-Methylphaseollin isoflavan (V) | 0.21 | 5.7 | −3.1 |
| (−)-7-*O*-Methylphaseollin (II) | 0.35 | 4.1 | −2.1 |
| Licoisoflavone B (VI) | 1.2 | 2.9 | −3.7 |
| (−)-Phaseollidin (III) | 1.6 | 2.8 | −3.9 |
| (−)-Clausequinone (XI) | 6.5 | 2.2 | −5.8 |
| (−)-Vestitol (IX) | 16 | 2.2 | −6.5 |
| | 9.3 | 2.7 | −6.0 NS[b] |
| | 3.4 | 4.7 | −4.7 |
| 2′-Hydroxyformononetin (XIX) | 14 | 10 | −1.1 |
| 2′-Hydroxygenistein (XVI) | 20 | 7.1 | −3.8 NS |
| | 27 | 3.6 | −2.7 |
| (−)-Medicarpin (XII) | 42 | 1.8 | −10.2 |
| | 43 | 9.6 | −3.4 NS |
| (−)-Maackiain (XIII) | 49 | 2.2 | −10 |
| | 50 | 1.8 | −12 |
| (+)-Pisatin (XIV) | 55 | 2.1 | −7.8 |
| Kievitone (XXIV) | 120 | 3.6 | −3.6 |
| Luteone (XXI) | 260 | 8.1 | −3.9 |

[a] The error limits are ($FD_{50}$ ÷ c, $FD_{50}$ × c).
[b] NS: slope not significantly different from zero at 5% level. All other slopes are significant.

ferent occasions (compare Tables 1 and 3) reflects the inherent scatter of data in such a behavioral bioassay.

The slopes of the regression lines given in Table 1 are given as fecal pellet count per log concentration. On average, the standard stimulated response was approximately 15 pellets. The slope values listed correspond to a reduction in feeding of between 4 and 20% of this average when the concentration of the compound is doubled.

Isoflavonoids which failed the $FD_{50}$ or the slope criteria for feeding-deterrent activity over the concentration range are listed in Table 2 together with their feeding responses at the highest concentration (200 $\mu$g/g). Compounds VII, XXII, and XXXI showed significant reduction in feeding at this concentration by the Wilcoxon's Rank sum test, and XXIII and X showed significant reduction in feeding at a lower concentration (Lane et al., 1985). These compounds, while

TABLE 2. EFFECT OF ISOFLAVONOIDS ON FEEDING OF *Costelytra zealandica* LARVAE: NO SIGNIFICANT DOSE–RESPONSE CORRELATION

| Compound | Feeding response[a] (%), 200 μg/g |
|---|---|
| Licoisoflavone A (VII) | 67[b] |
| (−)-Sativan (X) | 84 |
| Genistein (XV) | 80 |
| Biochanin A (XVII) | 76 |
| Formononetin (XVIII) | 79 |
| Wighteone (XX) | 69 |
| Angustone A (XXII) | 31[b] |
| Angustone B (XXIII) | 74 |
| (±)-Methylequol (XXV) | 98 |
| Dihydroformononetin (XXVI) | 175 |
| Coumestrol (XXVII) | 86 |
| 7-Deoxy-5′-hydroxycoumestrol (XXVIII) | 77 |
| Osajin (XXIX) | 76 |
| Pomiferin (XXX) | 110 |
| 4-Hydroxy-3-arylcoumarylquinone (XXXI) | 48[b] |

[a]Based on Hodges-Lehmann estimates, standard = 100.
[b]Significant reduction in feeding: $P < 0.05$ Wilcoxon's rank sum test.

probably active, do not show the steady reduction in feeding with increasing dose over the range tested that is characteristic of the compounds in Table 1. Licoisoflavone A (VII) has consistently shown a mild depression in feeding at all concentrations, while for XXII and XXXI the highest dose may be at the beginning of the response range.

In order to compare directly the effect of certain structural differences on the feeding response of *C. zealandica* larvae, sets of two or three homologous isoflavonoids were tested on the same day under the same conditions. The $FD_{50}$ values and slopes of dose–response lines for these comparative tests are given in Table 3. For comparison of active compounds in these sets, a parallel line analysis was used to check whether there were significantly different feeding responses and to provide a numerical measure of the difference. Parallel response lines were fitted to the Hodges-Lehmann estimates of the pellet counts for the compounds to be compared. The common slope was then checked for statistical significance. The *Y*-axis intercepts were compared using Fisher's LSD test (5% level), and the ratio of $FD_{50}$ values was calculated to give a measure of the relative activity. This analysis was not appropriate for comparisons involving compounds with an $FD_{50} > 1000$ and/or a positive or nonsignificant slope of the dose–response line.

All the compounds tested on this occasion (Table 3) gave similar results,

TABLE 3. COMPARATIVE EFFECT OF SELECTED ISOFLAVONOIDS ON FEEDING OF *Costelytra zealandica* LARVAE

| | Feeding response | | | | |
|---|---|---|---|---|---|
| Compound | $FD_{50}$ (μg/g) | $C$ (×, ÷) | Slope of dose-response line | Relative activity[a] | $C$ (×, ÷) |
| (−)-Medicarpin (XII) | 76 | 4.7 | −4.4 NS[c] | — | |
| (−)-Phaseollidin (III) | 5.3 | 4.0 | −5.1 | 1 | |
| (−)-Phaseollin (I) | 0.050 | 2.3 | −8.1 | 75 | 2.0 |
| 2′-Hydroxygenistein (XVI) | 380 | 4.9 | −5.1 | 1 | |
| Licoisoflavone A (VII) | 320 | 44 | −3.4 NS | — | |
| Licoisoflavone B (VI) | 2.7 | 1.9 | −7.8 | 68 | 2.2 |
| Genistein (XV) | >1000 | — | −2.5 NS | — | |
| 2′-Hydroxygenistein (XVI) | 97 | 2.3 | −6.0 | — | |
| Formononetin (XVIII) | NO[b] | — | +2.1 NS | — | |
| 2′-Hydroxyformononetin (XIX) | 18 | 390 | −1.2 NS | — | |
| (±)-Methylequol (XXV) | >1000 | — | −6.2 | — | |
| (±)-Vestitol (rac. IX) | 370 | 17 | −2.8 NS | — | |
| (±)-Medicarpin (rac. XII) | NO | — | +1.2 NS | — | |
| (+)-Vestitol (XXXII) | 460 | 23 | −3.2 NS | — | |
| (±)-Vestitol (rac. IX) | 35 | 3.1 | −6.4 | 1.6 NS | 2.2 |
| (−)-Vestitol (IX) | 43 | 2.5 | −7.6 | 1 | |
| Epirotenone (XXXIII) | 56 | 5.0 | −5.4 | 1 | |
| Rotenone (VIII) | 0.44 | 2.0 | −8.5 | 86 | 2.9 |
| Epirotenone (XXXIII) | 2.1 | 2.3 | −7.8 | 1 | |
| Rotenone (VIII) | 0.42 | 1.8 | −11 | 8.4 | 1.5 |
| Epirotenone (XXXIII) | 120 | 4.6 | −3.8 | 1 | |
| Rotenone (VIII) | 5.0 | 3.6 | −3.1 | 34 | 3.1 |

[a] Inverse ratio of $FD_{50}$ values for pairs of active compounds as determined by the parallel line analysis, expressed as 1 : n.
[b] NO: not obtainable, $FD_{50}$ not possible with compounds of positive slope.
[c] NS: not significant, slope not significantly different from zero at 5% level; activity difference not significant at 5% level.

TABLE 4. EFFECT OF SIMPLE CHROMENES ON FEEDING OF *Costelytra zealandica* LARVAE

| Compound | Feeding response | |
|---|---|---|
| | $FD_{50}$ (μg/g) | $C$ (×, ÷) |
| Precocene II (XXXV) | 2.4 | 1.7 |
| Precocene I (XXXIV) | 6.9 | 3.8 |
| 6-Acetyl-5-hydroxy-2′-2′-dimethylchromene (XXXVII) | 8.4 | 2.1 |
| Dihydroprecocene II (XXXVI) | 47 | 2.1 |

within the limits of error, to those obtained previously (Table 1 and 2). Thus, for example, phaseollidin (III) and phaseollin (I) gave comparable $FD_{50}$ and slope values, and (−)-medicarpin (XII) again gave a nonsignificant slope.

Table 4 records the $FD_{50}$ values and slopes of three simple chromenes and a chroman. In all cases, the data showed these compounds to have feeding-deterrent activity.

To simplify the discussion of each of the isoflavonoids, the isoflavone numbering system (see structure I) has been used throughout. Unless specifically indicated in the diagrams or in the text, the isoflavans and pterocarpans (reduced ring C compounds) have the same stereochemistry as phaseollin (I). The exceptions are (+)-pisatin (XIV) which has the opposite configuration to phaseollin at C-3 and C-4 and (±)-methylequol (XXV) which is a racemate. The isoflavanones, dihydroformononetin (XXVI) and kievitone (XXIV) were also obtained as racemates (Wong, 1975).

## DISCUSSION

The data included in this paper are the quantitative measures of a behavioral response of field-collected insects. In interpreting them as a whole, we have sought to identify trends. Some have emerged which are clearly supported by the statistical tests. Other apparent trends are less strongly supported by the analysis but still suggest a particular interpretation. We have attempted to find a common molecular basis for this pattern of results and consider a number of hypotheses in turn.

*Structural Correlations.* Of 36 isoflavonoids, including optical isomers, tested for feeding-deterrent activity towards *Costelytra zealandica* larvae, 18 were active. Phaseollin (I) and related compounds with a cyclic isoprenoid unit fused to ring B, and rotenone (VIII) are particularly active with a significant effect on feeding at concentrations below 1 μg/g. The feeding deterrent activity of rotenone (VIII) is of interest as it is well known as an insect toxin. Feeding

deterrent activity is not restricted to a particular isoflavonoid class. While all the pterocarpans tested (I–III and XII–XIV) showed feeding deterrent activity and neither of the coumestans tested (XXVII, XXVIII) showed activity, isoflavans, isoflavones, and isoflavanones occurred in both the "active" (Table 1) and "inactive," (Table 2) categories.

Many of the isoflavonoids showing insect feeding deterrent activity are also antifungal (Sutherland et al., 1980, Hart et al., 1983). However, a study of lupin isoflavones (Lane et al., 1985) has shown that the two activities do not always coincide and appear to have different structural requirements. Thus, 2′-hydroxygenistein (XVI) shows insect feeding-deterrent activity but is not antifungal, while the antifungal prenylisoflavone, wighteone (XX), does not show insect feeding-deterrent activity. Perrin and Cruickshank (1969) suggested a stereochemical basis for the antifungal activity of pterocarpans, but this was later rejected by Van Etten (1976), who found the antifungal activity of 17 isoflavonoids tested could not be correlated with a common three-dimensional shape. Whatever the structural requirements for the antifungal activity of isoflavonoids, further evidence that they are not identical with those responsible for insect feeding-deterrent activity is provided by the results reported here for 7-*O*-methylphaseollin (II) and rotenone (VIII). Both of these compounds show very high feeding-deterrent activity (Table 1), but both lack antifungal activity (R.A. Skipp, personal communication; Georgopoulos, 1977).

Two structural features that are characteristic of most of the highly active feeding deterrents are: (1) the presence of a 2′-oxy function, and (2) the occurrence of a 2,2-dimethyl-1,H-pyran (cyclic isoprenoid group) fused to ring B (I, II, IV, V). All the active isoflavonoids in Table 1 contain an oxy function at the 2′-position of ring B. Conversely, none of the 2′-deoxy compounds tested was active (Table 2). When pairs of homologous compounds were compared, the 2′-deoxy compounds, formononetin (XVIII), genistein (XV), methylequol (XXV), and wighteone (XX) were inactive (Table 2), whereas their 2′-hydroxy analogs, XIX, XVI, IX, and XXI, were active (Table 1). Direct comparative assays of several of these pairs proved less clear-cut (Table 3). The 2′-deoxy compounds genistein (XV), formononetin (XVIII), and methylequol (XXV) were again clearly inactive, and the difference between genistein (XV) and its 2′-hydroxy homolog (XVI) was confirmed. However, a difference between formononetin (XVIII) and 2′-hydroxyformononetin (XIX) and between (±)-methylequol (XXV) and (±)-vestitol (racemic IX) could not be unequivocally established as the 2′-hydroxy compounds on this occasion did not show a statistically significant dose–response slope. The cyclic ether homolog of (±)-methylequol (XXV), (±)-medicarpin (racemic XII) was also inactive, although (−)-medicarpin (XII) is a marginally active feeding deterrent (Table 1). These results highlight the difficulty of comparing inactive compounds with those of marginal activity, but the balance of evidence supports the view that the difference in activity between 2′-deoxy compounds and their 2′-oxy counterparts is real. Several inactive or

marginally active compounds do contain a 2′-oxy moiety (X, XXVII, XXVIII, XXII, XXIII, XXXI, VII), and the contrast between the inactivity of the coumestans (XXVII, XXVIII) and the active pterocarpans has already been noted. Thus, while 2′-oxygenation is a feature of all the active feeding deterrents, its occurrence does not always correlate with activity.

The second important structural feature is the presence of a ring B-fused cyclic isoprenoid moiety (2,2-dimethyl-1,H-pyran). The significance of this feature is highlighted by the high absolute activity of phaseollin (I) and its homologs and by the high relative activity of compounds I and VI compared to compounds III, XII and VII, XVI respectively (Table 3). Phaseollin with a cyclic isoprenoid group fused to ring B is much more active than (−)-medicarpin (XII) and 75 times more active than phaseollidin (III) with a noncyclic isoprenyl group. A similar tendency is seen when 2′-hydroxygenistein (XVI) is compared with the complex isoflavones containing isoprenyl (VII) and cyclic isoprenoid (VI) groups. Licoisoflavone B (VI) containing a ring B cyclic isoprenoid moiety is 68 times more active than 2′-hydroxygenistein (XVI) and the analog with a noncyclic isoprenyl group (VII) gave a nonsignificant dose response.

The occurrence of a cyclic isoprenoid moiety on the isoflavonoid molecule is not always associated with feeding-deterrent activity. Thus, angustone B (XXIII) shows only marginal signs of activity, and the ring A isoprenoids, osajin (XXIX) and pomiferin (XXX) are both inactive. On the other hand, of the highly active compounds, only rotenone (VIII) does not have a cyclic isoprenoid moiety fused to ring B, but rather, it contains an isoprenoid-derived furano group attached to ring A. The activity of this complex isoflavonoid is discussed below.

The high activity of the complex isoflavonoids prompted us to investigate the feeding deterrent activity of some simple 2,2-dimethylchromenes (Table 4). These compounds can be considered as partial structures to the ring B cyclic isoprenoid isoflavonoids. Precocene I (XXXIV) and precocene II (XXXV) are of particular interest as they have been found to act as antijuvenile hormones against a range of insects by cytotoxic action on the corpora allata (Bowers, 1976, 1981). The chromenes XXXIV, XXXV, and XXXVII all showed appreciable feeding deterrent activity, while the chroman XXXVI was less active. The chromene encecalin (analog of XXXIV, XXXV with R = $COCH_3$) has been reported as a feeding deterrent to *Heliothis zea* (Proksch and Rodriguez, 1983). The feeding-deterrent activity of these compounds may reflect structural similarities to the cyclic isoprenoid isoflavonoids or may have an independent basis.

*Modes of Action: Reactive Centers.* In considering and interpreting the pattern of results discussed thus far, we have not found any direct parallels with previous studies of insect feeding deterrents. Oxygen functionality and oxidation state have been found to be significant factors in studies of the insect feeding-deterrent activity of phenolics. Elliger et al. (1980) found the inhibition of growth of *Heliothis zea* larvae caused by the presence of a range of flavonoids

in their food was associated with ring B *ortho*-dihydroxylation. Norris (1977) found that the presence of a carbonyl and an adjacent hydroxyl in compounds such as 5-hydroxynaphthoquinone, quercetin, and kaempferol gave high feeding inhibition toward *Scolytus multistriatus*. He concluded that there was a strong positive correlation of antifeeding activity with the degree of oxidation state of ring C of the flavonoids and also with the intramolecular hydrogen-bonding capability of the molecule. Any correlation between the oxidation state of the isoflavonoids and the feeding response of *Costelytra zealandica* does not follow the same pattern, since it is the reduced isoflavans and pterocarpans which are more active than the highly oxidized isoflavones, coumestans, and quinones. Sites for intramolecular hydrogen bonding are available around the carbonyl region in isoflavones, and it could be argued that this factor accounts for the higher activity of the 2′-hydroxyisoflavones (VI, XVI, XIX, XXI). However, such a mechanism cannot account for the activity of the 2′-hydroxyisoflavans (IV, IX) and pterocarpans (I–III, XII, XIII). Moreover, the presence of a strongly intramolecularly hydrogen-bonded 5-hydroxy group in genistein (XV) and its homologs (VI, VII, XVI, XVII, XX–XXIII) does not seem to confer any special increase in feeding-deterrent activity. Therefore, we conclude that our results with *C. zealandica* feeding are not explained by the relative location of oxygen functionality alone.

Reactive centers of the electron donor–acceptor type in close proximity, as typified by the intramolecular hydrogen-bonding sites discussed above, have been identified as the key structural feature for the activity of quinoid and flavonoid (Norris, 1977) and isodon diterpenoid (Kubo and Genjian, 1981) feeding deterrents. The mode of action of such compounds as juglone, polygodial, and warburganal has been attributed to the interaction of the reactive electrophilic center with the sulfhydryl site of a receptor macromolecule (Norris, 1976; Singer et al., 1975, Ma, 1977). If such binding to sulfhydryl sites were important in the gustatory response of *C. zealandica,* then the quinone isoflavonoids (XI, XXXI) would be expected to be especially deterrent, but this was not observed. Further, such structural features are not evident in the most highly active isoflavonoids (I, II, IV–VI, VIII).

*In Situ Activation.* If isoflavonoid feeding deterrents are not characterized by the presence of reactive electrophilic centers, they may be precursors of reactive species generated in situ which could react with a receptor macromolecule. A number of possibilities are suggested by the literature. Recently, Bakker et al. (1983) have reported the photoactivation of several pterocarpan phytoalexins to free-radical species which deactivated the enzyme glucose-6-phosphate dehydrogenase in an *in vitro* assay system. Other photoactive compounds such as furocoumarins have been reported as insect feeding deterrents (Yajima et al., 1977). The pertinence of a photoactivation mechanism to resistance to root-feeding larvae is not clear. In any case, the pattern of photoactiv-

ities reported by Bakker et al. (1983) [pisatin (XIV) > phaseollin (I); medicarpin (XII) inactive] differs from the pattern of feeding deterrent activity for these compounds with *C. zealandica* (Table 1), and there was no exposure of materials to direct sunlight in the feeding assay procedure. While a photoactivation mechanism seems unlikely, the involvement of free radical species in the insect response to isoflavonoids cannot be excluded.

The reported oxidation of the cyclic isoprenoid moiety of precocene (XXXIV) to a reactive epoxide in insect corpora allata (Pratt et al., 1980) indicates another mode of bioactivation of these molecules. The reduced activity of dihydroprecocene II (XXXVI) compared to precocene II (XXXV) (Table 4) suggests that the presence of the reactive double bond favors feeding-deterrent activity, but this feature is clearly not essential for activity.

The oxidative formation of reactive *ortho*-quinone-methide species has been invoked by Jurd et al. (1979) to explain the pattern of insect toxicity and chemosterilant activity of a range of benzylphenols. A similar process has been suggested to account for the anti-juvenile hormone activity of a prenyl phenol analogous to precocene II (XXXV) (Bowers et al., 1982), and valence isomerization to the corresponding quinone-methide has been suggested as a mode of activation of the precocenes (XXXIV, XXXV) (Bowers, 1981). Chemical evidence has recently been obtained for the formation of *para*-quinone-methides from 7-hydroxyflavans (Attwood et al., 1984). Of the isoflavonoids studied here, those with a benzylic proton *ortho* or *para* to a phenolic hydroxyl, or with a fused 2,2-dimethyl-1,4-pyranyl ring, can be considered as quinone-methide precursors. However, the presence or absence of these structural features alone does not correlate with the observed pattern of feeding-deterrent activity (e.g., compare XVI, Table 1; and XX, Table 2).

*Stereochemistry.* From this discussion it is clear that reactivity factors such as intramolecular hydrogen bonding, redox potentials, and the formation of reactive species could be envisaged as accounting for the feeding-deterrent activity of some of the active isoflavonoids but not in a consistent way. If we make the assumption that there is a common basis to the observed pattern of structure–activity relationships, it cannot be found in such reactivity considerations alone. This has led us to consider the stereochemical similarities of the compounds, in particular phaseollin (I) and rotenone (VIII). These are both highly active deterrents, have few reactive centers, and have rather rigid polycyclic structures with relatively few stable conformations. However, rotenone (VIII) does not contain a ring B cyclic isoprenoid moiety characteristic of the other highly active feeding deterrents, and the alignment of the 2′-oxygen is quite different from that in the pterocarpans. Further, the absolute stereochemistry at C-3 is opposite to that for (−)-pterocarpans such as phaseollin (I) (Wong, 1975).

In a study of the bifunctional binding of rotenone with mitochondrial NADH

dehydrogenase, Yaguzhinskii and Kolesova (1975) concluded that the polar ring B and the carbonyl group determine the interaction of the molecule with the binding site. That this binding is stereospecific is indicated by the fact that epimeric compounds are inactive, which the authors attributed to the inability of the carbonyl to lie in the proper region at the phase boundary. The nature of the furanoid moiety on ring A seemed to be less specific. Rotenone can thus be considered as a nonplanar molecule with lipophilic and polar ends and containing a central dipole (Figure 2). This stereospecific enzyme binding model raises the possibility that there might be a stereochemical basis for the observed pattern of feeding deterrent activity, albeit less specific than for NADH dehydrogenase inhibition.

We suggest that the common feature for the active feeding deterrents is that they have, or can adopt, a similar nonplanar molecular shape with a similar arrangement of polar and lipophilic groups. The molecular structures of phaseollin (I) and rotenone (VIII) have been the subject of both X-ray crystallographic (DeMartinis et al., 1978; Arora et al., 1975) and NMR studies (Pachler and Underwood, 1967; Perrin et al., 1972; Carlson et al., 1973) which indicated a consistent shape in both the solid and solution phases. Both Dreiding models and computer generated plots (PLUTO; Motherwell, 1976) (Figure 2) suggest that when the molecules are appropriately aligned their stereochemistry is com-

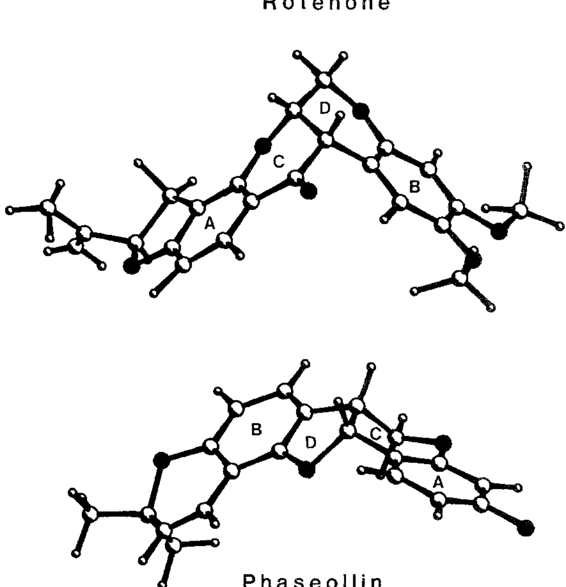


FIG. 2. Projections of molecular structures of rotenone and phaseollin from X-ray crystal coordinates.

parable. The ring A of phaseollin can be aligned with the ring B of rotenone so that the dihydrofuran oxygen (2′-oxygen) occurs within the same region as the important carbonyl of rotenone. The cyclic isoprenoid group is then in a similar region to the dihydrofuran ring of rotenone and the phenolic hydroxyl of phaseollin in a similar region to the two methoxyl groups of rotenone. This comparison suggests a basis for the importance of the isoflavonoid 2′-oxy function, which could serve a similar role to the rotenone carbonyl in binding to a macromolecule, and the importance of the cyclic isoprenoid group on ring B in defining a lipophilic region of the molecule. Of the active chiral isoflavonoids initially tested (Table 1), all the isoflavans and pterocarpans, with the exception of (+)-pisatin (XIV) are of the same absolute stereochemistry as (−)-phaseollin and are able to adopt a similar relative arrangement of 2′-oxygen and aromatic rings. (+)-Pisatin (XIV) can be aligned similarly to rotenone with the angular hydroxyl (on C-3) in the vicinity of the carbonyl group.

The inactive 2′-deoxyisoflavones (XV, XVII, XVIII, XX) and isoflavan (XXV) cannot be aligned with a similar arrangement of groups, nor can the planar coumestans (XXVII, XXVIII). The 2′-hydroxyisoflavones can be aligned to some extent by rotation of ring B out of the plane of rings A and C, and most of these compounds are active. The high activity of licoisoflavone B (VI) may be associated with the possibility of aligning both the 2′-hydroxy and the cyclic isoprenoid group similarly to phaseollin. In the inactive prenyl homolog (XXIII), the molecule no longer has defined polar and lipophilic ends. The contrast between the activity of vestitol (IX) and the inactivity of sativan (X) may be associated with the steric effect of the 2′-*O*-methyl, as 2′-*O*-methyl phaseollin isoflavan (V) also appears to be appreciably less active than its 2′-hydroxy counterpart (IV) (Table 1). The activity of the simple chromenes (XXXIV, XXXV, XXXVII) can be rationalized if they are considered to be partial phaseollin-like structures corresponding to the ring B and cyclic isoprenoid regions. Thus, the stereochemical binding model does appear to be consistent with the observed pattern of results discussed so far.

Some feeding assays were carried out, using available stereoisomers, to investigate the importance of stereochemistry to feeding-deterrent activity of *C. zealandica* larvae. The activity of rotenone was compared on several occasions with that of epirotenone (XXXIII) which has the opposite configuration at the junction of rings C-D. The NMR data (Methods) indicate an essentially enantiomeric relationship between rings C and D of rotenone and epirotenone and hence an opposite sense of folding. The results (Table 3) show that epirotenone is significantly less active than rotenone, although the difference is considerably less than for enzyme inhibition (epirotenone 1000-fold less active, Yaguzhinskii and Kolesova, 1975). Further, (+)-vestitol (XXXII) appeared to be less active in a comparative assay with (−)-vestitol (IX) and synthetic racemic (±)-vestitol, although this difference was statistically not significant. Thus stereochemical considerations appear to be a factor in isoflavonoid feeding deterrent activ-

ity, and the relative activity of these stereoisomers can be related to how closely they approximate a phaseollin-like shape.

## CONCLUSIONS

Kojima and Kato (1981) concluded in their study of clerodin feeding deterrents that a definite steric environment around an active binding center is required for high activity. Similarly, the active isoflavonoids (including rotenone) can be considered as defining, in terms of stereochemistry, polarity, and lipophilicity, the environment of the key oxygen. Further elucidation of the stereochemical and possible reactivity factors involved in the feeding-deterrent activity of isoflavonoids awaits the availability of suitable synthetic analogues.

The sensitivity of *C. zealandica* larvae to phaseollin, its analogs, and rotenone is remarkable. While our results suggest a common stereochemical basis for this phenomenon, the biochemical mechanism of this behavioral response remains to be established. The ability of these root-feeding larvae to detect and avoid low concentrations of the insect toxin, rotenone, normally found in roots of several tropical legumes (e.g., *Dalbergia* and *Tephrosia* spp.) is intriguing for its evolutionary implications. *C. zealandica* is indigneous to New Zealand flora, although pterocarpans and isoflavans are present (Briggs et al., 1975; Ingham, 1983).

The wide differences in activity we have found for isoflavonoid feeding deterrents of the same structural class emphasize the need for quantitative methods and detailed analysis in determining the ecological role of plant secondary metabolites. Thus, phaseollin is likely to play a significant role in defense against insect attack at much lower plant concentrations than the simple pterocarpan, medicarpin, and the insect resistance of legumes may relate to the structures of the endogenous isoflavonoids. This raises the possibility of modifying the isoflavonoid biosynthetic pathway to enhance the resistance of a susceptible legume toward the feeding of *C. zealandica* larvae.

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# EFFECT OF GAMMA IRRADIATION ON ALLELOPATHIC POTENTIAL OF *Sorghum bicolor* AGAINST WEEDS AND NITRIFICATION

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**Abstract**—The effect of low doses of gamma irradiation on the allelopathic potential of *Sorghum bicolor* against weeds and nitrification was investigated. The results revealed that all test doses (500, 1000 and 1500 rad) significantly increased the allelopathic activity of root exudates, aqueous extracts, and decaying residues aganst seed germination and seedling growth of *Amaranthus retroflexus*. The results also indicated that all test doses stimulated the allelopathic potential of aqueous extracts and decaying residues against nitrification activity. The possible application of this approach in biological control is briefly discussed.

**Key Words**—Allelopathy, gamma irradiation. *Sorghum bicolor*, *Amaranthus retroflexus*, nitrification, biological control.

## INTRODUCTION

Recently, research activities on the allelopathy of crops have accelerated with the aim of using this phenomenon in biological control (Rice, 1979; ALsaadawi and Rice, 1983; Putnam, 1983). One approach of research in this aspect is to utilize a companion or rotational crop which may not be harvested in the cropping sequence, but may provide toxicity to weeds upon decaying of its residues (Barnes and Putnam 1983; Putnam et al., 1983; Gliessman, 1983). A second approach is to screen the accessions or cultivars of allelopathic crops to select the most allelopathic ones (Putnam and Duke, 1974; Fay and Duke, 1977; Leather, 1983).

Another possible approach is to maximize the production of allelopathic



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agents by several environmental factors including irradiation. Rice (1974) and recently Putnam (1983) reviewed some of these factors. However, efforts were limited and not all studies were carried out with possible use of allelopathy in biological control in mind.

The goal of this study was to test the possible effects of low doses of gamma irradiation on the allelopathic potential of *Sorghum bicolor* against weeds and nitrification. Inhibition of nitrification was reported to be very important in agricultural ecosystems (Huber et al. 1977). These workers indicated that inhibition of nitrification may considerably increase the efficiency of food production, reduce energy requirements for growing crops, decrease the incidence of plant disease, and reduce the pollution potential of nitrogen fertilizers.

## METHODS AND MATERIALS

### *Effect of Gamma Irradiation on Allelopathy of* Sorghum bicolor *against* Amaranthus retroflexus

*Root Exudate Bioassay.* Air-dried seeds of *Sorghum bicolor* were irradiated with 0, 500, 1000, and 1500 rad of gamma rays using a gamma cell 40 (Atomic Energy of Canada Ltd) equipped with $^{137}Cs$ source. The dose rate was 126 R/min as measured by Fricke dosimetry. Five seeds of each treatment were planted separately in 8-cm-diameter plastic pots containing 260 g of sterilized acid-washed sand. Pot controls were run in the same manner but without sorghum seeds. The pot control was made to confirm the allelopathic activity of root exudates of the sorghum cultivar studied. The growth conditions of this and all subsequent experiments were $26 \pm 2°C/24 \pm 2°C$ day/night temperature, 14-hr/photoperiod and 1900 ft-c light intensity. The plants were watered daily with distilled water on days 1, 2, 3, and 4 and with half-strength Hoagland's solution (Hoagland and Arnon, 1950) on the remaining days.

Ten days after planting, root exudates were collected by suction-filtering each pot using a gentle vacuum. Thirteen milliliters of root exudates of each treatment were added to Petri dishes (10 cm diameter); each containing 60 g of acid-washed sand and 25 seeds of *Amaranthus retroflexus*. Seed germination and radicle and hypocotyl lengths were measured 7 days after planting.

*Aqueous Extract Bioassay.* Groups of air-dried seeds of *Sorghum bicolor* were irradiated with the same doses of gamma rays as mentioned previously. The seeds were planted in plastic pots containing 7 kg of loamy soil under greenhouse conditions. After two months, the plants were harvested and used as test materials for this and all subsequent experiments.

The water extracts of *Sorghum bicolor* were prepared following the method of Rice (1972). Ten grams fresh weight of each treatment were boiled separately in 100 ml distilled water for 10 min, then ground in an electrical blender for 10

min. The extracts were suction-filtered through filter paper (15–40 μm pore size). The filtrate was made up to the original volume and centrifuged for 10 min at 15000 rpm for further purification. The supernatant was adjusted to pH 6.0.

The test solutions were made by diluting the purified solution to 1:1 (vv, extract–Hoagland's solution). Control solutions consisting of distilled water diluted 1:1 with Hoagland's solution were used to test the alleopathic activity of sorghum extract.

The biological activities of aqueous extracts were determined using a sand culture technique. Twenty-five seeds of *Amaranthus retroflexus* were planted per pot containing 330 g of acid-washed sand. After planting, each pot received 70 ml of a selected solution. The pots were arranged in a randomized complete block design with six replications for each treatment. Twice a month, each pot received 20 ml of a selected solution, otherwise, they were watered with equal amounts of 0.5 strength Hoagland's solution alternated with equal amounts of distilled water. After two weeks, seed germination was recorded and the plants were thinned to the two largest per pot. After one month, oven-dry weights of roots and tops were determined.

*Decaying Residue Bioassay.* Twenty-five seeds of *Amaranthus retroflexus* were planted in each of 20 plastic pots each containing 500 g of loamy soil mixed with 3 g of a selected treatment of sorghum powder. There were four series with five replications for each series. The peat moss control series was made by adding 3 g of peat moss to each pot, instead of sorghum powder, to keep the organic matter the same. This control was basically run to test the allelopathic activity of decaying residue of the sorghum cultivar tested. All pots were watered equally with distilled water. Seed germination was recorded two weeks after seeding, after which time the plants were thinned to the three largest per pot. Dry weights of roots and tops of the test cultivars were taken four weeks after initiation of the experiment.

*Effect of Gamma Irradiation on Allelopathic Potential of* Sorghum bicolor *against Nitrification Activity.*

*Aqueous Extract Bioassay.* Soil for nitrification evaluation was collected to a depth of 10 cm in August 1984. The soil was mixed thoroughly, air-dried, and passed through a 2-mm sieve. One hundred grams of soil were added to a 100-ml plastic beaker. Each beaker received 12 ml of an aqueous solution containing 0.0944 g of $(NH_4)_2SO_4$ to make the concentration of $NH_4$ nitrogen added 200 ppm for the distilled water control. Treatments were run similarly except distilled water was replaced by a 10% aqueous plant extract of selected test material. The experiment was conducted in an incubator in darkness and at a temperature of 28°C. The experiment was arranged in a randomized complete block design with five replications.

The amounts of $NH_4$ nitrogen and $NO_3$ were determined every two days using the MgO–Devarda alloy method (Bremner, 1965).

*Decaying Residue Bioassay.* One gram of air-dried powder of sorghum test material was mixed in a 100-ml plastic beaker. A peat moss control was made by mixing 1 g of peat moss instead of the sorghum test powder with 100 g of soil. Each beaker received 0.0944 g of $(NH_4)_2SO_4$ to make the $NH_4$ nitrogen concentration added 200 ppm. The experimental design, growth conditions, and $NH_4$ nitrogen and $NO_3$ nitrogen measurements were carried out in the same manner as mentioned previously.

## RESULTS

*Effect of Gamma Irradiation on Allelopathic Potential of* Sorghum bicolor *against* Amaranthus retroflexus.

*Root Exudates Bioassay.* Sorghum root exudates of all treatments, except 0 rad on hypocotyl, caused significant reduction to radicle, hypocotyl, and seedling growth of *A. retroflexus* when compared to the pot control (Table 1). Root exudates of the 500-, 1000-, and 1500-rad treatments significantly inhibited radicle, hypocotyl, and seedling growth as compared with the untreated one. However, root exudates of the 1000-rad treatment caused more inhibitory effect to hypocotyl and seedling growth of *A. retroflexus* than the root exudates of the other sorghum treatments.

Seed germination was appreciably inhibited by root exudates of the 500- and 1000-rad treatments and slightly by the 0- and 1500-rad treatments.

*Aqueous Extract Bioassay.* Aqueous extracts of all sorghum treatments significantly reduced root, top, and seedling growth of *A. retroflexus* (Table 2). Aqueous extracts of the 500-, 1000-, and 1500-rad treatments significantly reduced root, top, and seedling growth when compared to the untreated one. The

TABLE 1. EFFECT OF GAMMA IRRADIATION ON ALLELOPATHIC POTENTIAL OF SORGHUM ROOT EXUDATES AGAINST *Amaranthus retroflexus*

| | Mean length (mm)[a] | | | |
|---|---|---|---|---|
| Treatment | Radicle | Hypocotyl | Seedling | Germination (%) |
| Pot control | 19.91a | 20.86a | 40.77a | 98 |
| 0 rad control | 16.47b | 19.48a | 35.95b | 92 |
| 500 rad | 12.52c | 16.81bc | 29.33c | 84 |
| 1000 rad | 12.09c | 15.17c | 27.26d | 84 |
| 1500 rad | 12.24c | 17.27b | 29.51c | 88 |

[a] Average of at least 25 seedlings. Means within column followed by the same letters are not significantly different at 0.05 level according to Duncan's multiple-range test.

TABLE 2. EFFECT OF GAMMA IRRADIATION ON ALLELOPATHIC POTENTIAL OF SORGHUM AQUEOUS EXTRACT AGAINST *Amaranthus retroflexus*

| | Dry weight (mg)[a] | | | |
|---|---|---|---|---|
| Treatment | Root | Top | Seedling | Germination (%) |
| Distilled $H_2O$ (control) | 15.4a | 52.2a | 67.6a | 92 |
| 0 rad (control) | 11.2a | 25.4b | 36.6b | 65 |
| 500 rad | 5.7c | 16.9d | 22.6d | 61 |
| 1000 rad | 7.6c | 21.6c | 29.2c | 74 |
| 1500 rad | 9.6b | 19.9d | 29.5c | 72 |

[a] Average of at least 20 seedlings. Means within column followed by the same letters are not significantly different at 0.05 level according to Duncan's multiple-range test.

aqueous extract of the 500-rad treatment was less inhibitory to root, top, and seedling growth of *A. retroflexus* than the aqueous extracts of the other treatments.

Seed germination was appreciably inhibited by the aqueous extracts of all sorghum treatments. The aqueous extracts of the 500-rad treatment slightly inhibited seed germination, while aqueous extracts of the 1000- and 1500-rad treatments slightly stimulated germination.

*Decaying Residue Bioassay.* Root and seedling growth of all sorghum treatments and top growth of all sorghum treatments except the 0-rad treatment were significantly reduced when compared to the peat moss control (Table 3). Decaying residues of the 500-, and 1000-rad treatments revealed the greatest inhibitory activity to root and top growth of *A. retroflexus*, respectively.

Seed germination was significantly reduced by decaying residues of the 0-, 500-, and 1500-rad treatments in comparison to the peat moss control. De-

TABLE 3. EFFECT OF GAMMA IRRADIATION ON ALLELOPATHIC POTENTIAL OF SORGHUM RESIDUE AGAINST *Amaranthus retroflexus*

| | Mean dry weight (mg)[a] | | | |
|---|---|---|---|---|
| Treatment | Root | Shoot | Seedling | Germination (% of control) |
| Peat moss (control) | 75.7a | 210.1a | 285.8a | 100 |
| 0 rad (control) | 38.0a | 216.3a | 254.3b | 76 |
| 500 rad | 26.0c | 125.7c | 151.7d | 66 |
| 1000 rad | 34.7b | 116.8c | 151.5d | 90 |
| 1500 rad | 34.3b | 147.2b | 181.5c | 77 |

[a] Average of at least 20 seedlings. Means within column followed by the same letters are not significantly different at 0.05 level according to Duncan's multiple-range test.

caying residues of the 500-rad treatment slightly inhibited seed germination, whereas the 1000-rad treatment slightly stimulated germination when compared to decaying residues of untreated plants.

*Effect of Gamma Irradiation on Allelopathic Potential of* Sorghum bicolor *against Nitrification.*

*Aqueous Extract Bioassay.* Aqueous extracts of all sorghum treatments significantly inhibited nitrification when compared to the distilled water control (Figure 1). There was no clear trend of differences in nitrification inhibition in relation to aqueous extracts of different sorghum treatments and in relation to periods of incubation. The result also indicated that the ammonium remaining was highly negatively correlated with the $NO_3$ produced in all treatments.

*Decaying Residue Bioassay.* Decaying residues of all test doses, including 0-rad, significantly reduced the nitrification process when compared to the peat moss control (Figure 2). The reduction became more pronounced at the sixth day of incubation. Decaying residues of the 500- and 1000-rad treatments significantly inhibited nitrification at 8 and 10 days incubation. There were highly

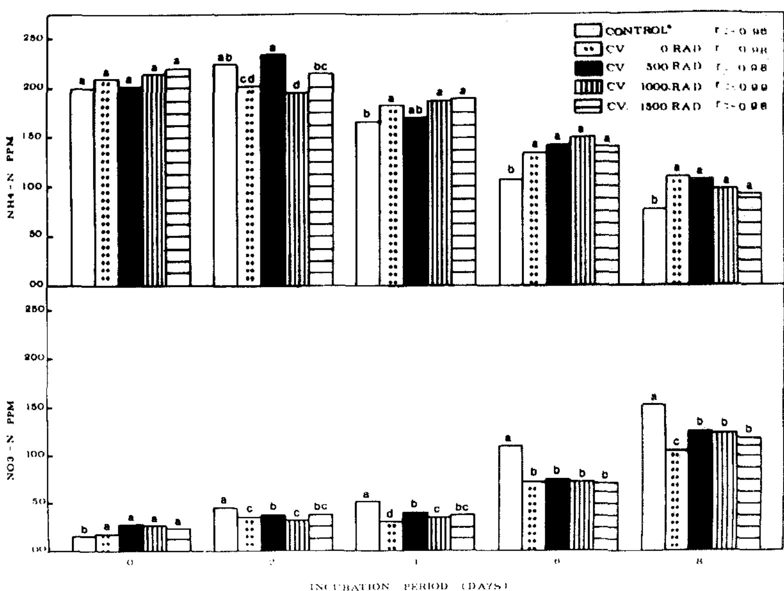


FIG. 1. Effect of gamma irradiation on the allelopathic potentiality of sorghum aqueous extract against nitrification. Means within each incubation period sharing the same letter are not significantly different at 0.05 level according to Duncan's multiple-range test. *Distilled water control.

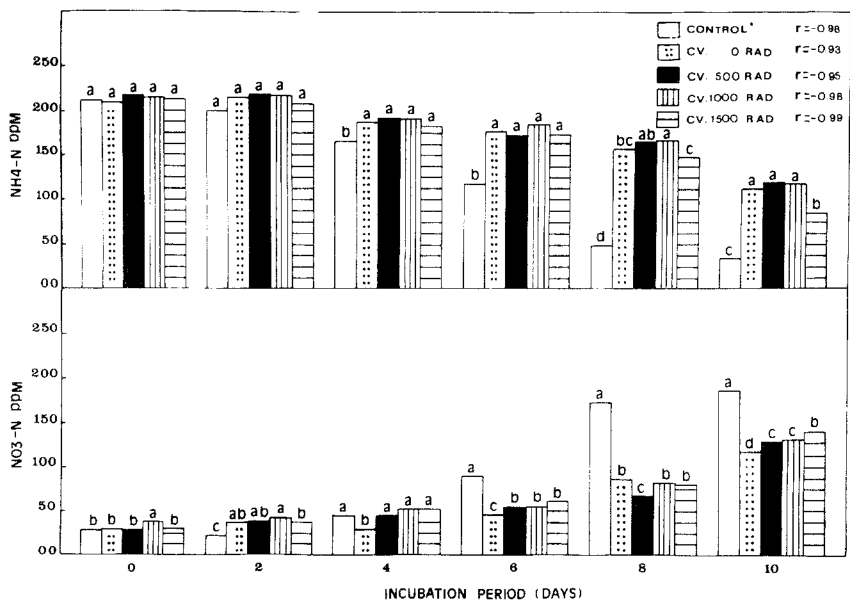


FIG. 2. Effect of gamma irradiation on the allelopathic potentiality of sorghum residues against nitrification. Means within each incubation period sharing the same letter are not significantly different at 0.05 level according to Duncan's multiple-range test. *Peat moss control.

significant negative correlations between the ammonium remaining and the nitrate produced in all treatments.

## DISCUSSION

The effect of radiation on phytotoxin production in plants was reported by several investigators (Frey-Wyssling and Babler, 1957; Fomenko, 1968; Koeppe et al., 1969; Del-Moral, 1972). Such an effect frequently leads to an increase in the production of phytotoxins. The present study clearly revealed that low doses of gamma irradiation stimulate the allelopathic potentiality of *Sorghum bicolor*. The inhibitor effects were shown in almost all experiments of the study; however, this inhibitory action is found to be more against *A. retroflexus* than against nitrification. No attempts were made to determine the qualities and quantities of the phytotoxin(s); however, Riov et al., (1968) and Koeppe et al., (1970) clearly demonstrated that several phenolic compounds increased drastically in plants after exposure to different types of ionizing radiations. Such radiation was found to stimulate the enzyme phenylalanine ammonia lyase (PAL)

which is considered a key enzyme in the metabolism of phenolic compounds (Riov et al., 1968).

The results indicated that gamma irradiation increased the allelopathic activity of root exudates and aqueous extracts. This suggests that at least some of this increment occurred in water-soluble phytotoxin(s) which are known to be important from an ecological point of view. Subsequent work on the decaying residues substantiated our results in that low doses of gamma irradiation also caused increases in the allelopathic activity of *sorghum* residues. This result is particularly important from a practical point of view for the following reasons: (1) the residue represents one of the major sources of phytotoxins, and (2) it can be used in no-tillage cropping systems to reduce soil erosion and improve water retention (Putnam, 1983).

It is noteworthy that stimulation of allelopathic activity can be achieved by low doses of gamma irradiation, since these doses are considered as stimulatory doses rather than mutagenic in *S. bicolor* (Berzino and Kawshainsky, 1975).

Our results indicated that low doses of gamma irradiation may prove a useful tool in increasing the allelopathic potential of some crops against weeds and the nitrification process without causing any genetic damage to the crops. However, further investigation is required before suggesting any effective methodology.

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# *cis*-VACCENYL ACETATE AS AN AGGREGATION PHEROMONE IN *Drosophila melanogaster*

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**Abstract**—Pentane extracts of mature *Drosophila melanogaster* males substantially increased the attractiveness of food odors to both males and females in a wind-tunnel olfactometer. Extracts of females caused no such increase. An active component of the extract was isolated and identified as (*Z*)-11-octadecenyl acetate (*cis*-vaccenyl acetate, cVA), and synthetic cVA was active in bioassay. Hydrolysis of the ester linkage or movement of the double bond to the 9 position destroyed the activity. Mature virgin males released cVA into their feeding vials, and amounts of synthetic CVA equal to that released per male caused significant bioassay responses. Females, which were known to receive cVA from males during copulation, were found to emit relatively large amounts of the ester into their feeding vials within 6 hr after mating. cVA had been demonstrated previously to be a close-range pheromone in *D. melanogaster*, discouraging males from courting other males or recently mated females; it now appears to have a longer-range function as well.

**Key Words**—Diptera, Drosophilidae, *Drosophila melanogaster*, pheromone, (*Z*)-11-octadecenyl acetate, *cis*-vaccenyl acetate.

## INTRODUCTION

Pheromonal involvement in the close-range courtship behavior of *Drosophila melanogaster* has been intensively studied in recent years (e.g., Tompkins and Hall, 1981a, b; Jallon et al., 1981; Antony and Jallon, 1982; Jallon, 1984). However, less is known about the mechanism by which flies come together under field conditions so that courtship can take place. Since the flies are attracted by odors from food sources (Kellogg et al., 1962) and since mating and oviposition



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take place at the feeding site (Spieth, 1974), a role for pheromones in aggregation might not be expected. Yet, in another fruit fly, *D. virilis*, which also mates at its feeding site, mature males produce an aggregation pheromone which attracts flies of both sexes (Bartelt and Jackson, 1984). This study was undertaken to determine whether *D. melanogaster* has an analogous pheromone system. An aggregation response was demonstrated, and (Z)-11-octadecenyl acetate, also known as *cis*-vaccenyl acetate (cVA), which is produced only by males, was found to be a major pheromone component.

## METHODS AND MATERIALS

*Flies and Extracts.* Wild-type flies of the Canton S strain were used for this research. They were reared on Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, North Carolina). To prepare extracts for biossay, flies were separated by sex when 0–1 day old (thus were predominantly virgin), and they were extracted with pentane in a Soxhlet apparatus for 8 hr when 4–10 days old. Aliquots of extract were fractionated by polarity on open columns of silicic acid. Elution solvents were hexane; 2.5%, 7.5%, and 25% ether in hexane; and ether. Further purification of active fractions was accomplished by preparative GLC.

To analyze flies for cVA and hydrocarbons, groups of 50 flies, separated by sex shortly after emergence, were placed in fresh rearing vials for various lengths of time (from 1 to 28 days). The flies were then extracted by soaking in hexane at 40°C for 24 hr. The extract and two hexane rinses of the flies were combined, passed through an open column of silicic acid with 10% ether in hexane, and reduced in volume for GLC analysis. Components were quantitated by GLC relative to an internal standard, nonadecane. There were two or three replications for each age group.

To study the emission of compounds from adult flies, the materials deposited in their rearing vials were analyzed. Twenty-five virgin males, 25 virgin females, or 10 mated females were placed in 30-ml glass rearing vials with fresh diet medium and allowed to feed for a set period of time (2 days or 6 hr). The mated females were transferred to the fresh vials immediately upon termination of copulation. After the flies were removed, the vials were rinsed for ca. 15 sec 3 times with 2-ml aliquots of hexane; then the extracts were combined, reduced in volume under $N_2$, and analyzed by GLC (2-m × 2-mm ID glass column packed with 3% Dexsil 300 on 100/120 Chromosorb WHP). Nonadecane, added to the samples after combining the rinses, served as the quantitative internal standard. The identification of cVA in samples was confirmed by coinjection with synthetic cVA on a 30-m × 0.25-mm ID Durabond DB-1 capillary column. By GLC, these extracts contained very little besides *D. melanogaster* hydrocarbons and, when relevant, cVA. The hydrocarbon profiles were always in excellent agreement with direct extracts of flies of the appropriate age and sex.

When 10-$\mu$g aliquots of nonadecane and cVA, in 10 $\mu$l of hexane, were added to fresh rearing vials, the recovery of nonadecane was 32 $\pm$ 7% (SD) after 2 days, and the recovery of cVA was 31 $\pm$ 6%, measured by GLC relative to an internal standard, heneicosene, added to the vial rinses. It is unknown to what extent penetration of the samples into the medium reduced recovery by extraction, but even after 2 days, the ester was recovered to the same extent as nonadecane. Thus the unsaturated ester did not break down any faster than the very stable alkane. The measured amounts of fly compounds must be considered as the *minimum* quantities released by the flies.

*Bioassay.* The bioassay procedure and equipment were described in detail previously (Bartelt and Jackson, 1984). Briefly, ca. 1000 flies 0–2 days old were added to a wind-tunnel olfactometer ca. 2 hr before tests were to begin. The olfactometer was large enough to allow free flight and was kept at 25–28°C. Although the number of flies in the olfactometer was fairly large, only ca. 10–20 flies were typically in flight in the olfactometer at any instant. Bioassay chemicals were applied to filter paper strips inserted around the openings of 30-ml glass vials. The "food" treatment was 0.5 ml of fresh rearing medium, inoculated with yeast 24 hr before the test, placed in the bottom of a bioassay vial. Two test vials, with treatments to be compared, were placed on the floor of the olfactometer, in the upwind end. When an experiment included more than two treatments, they were tested in pairs, in all possible combinations (a balanced incomplete block design). Responses included upwind casting flight, landing, and walking into the vial. After 3 min, the vials were capped and removed and the captured flies sexed and counted. Tests were separated by 7–10 min, and up to 30 tests could be run in a day with one group of bioassay flies. Analysis of bioasssay counts was done by the method of Yates (1940). The data were transformed to the $\log(X + 1)$ scale before analysis to stabilize variance. Thus ratios between catches for different treatments were more relevant than differences.

## RESULTS AND DISCUSSION

*Bioassays with Crude Extracts.* Initial attempts to demonstrate an aggregation pheromone in *D. melanogaster* were patterned after previous experiments with *D. virilis* (Bartelt and Jackson, 1984). With *D. virilis*, hexane extracts of male flies were highly attractive in bioassay flies which had been starved for at least 8–10 hr. With *D. melanogaster*, however, such extracts were not attractive, even when the bioassay flies were starved as long as 24 hr. Nevertheless, the relationship between bioassay response and food deprivation in *D. virilis* led us to test *D. melanogaster* extracts as coattractants of fermented food, and these experiments were successful. For example, the combination of food + male extract was about four times more attractive than food alone, the

mean catches being 9.8 and 2.5, respectively ($N = 56$, $P << 0.001$, 1 male equivalent/test).

The extracts of virgin females did not significantly increase the attractiveness of food. In one experiment the mean catches for food, food + female extract, and food + male extract were 0.8, 0.9, and 5.4, respectively ($N = 24$). Thus, as in *D. virilis*, there was evidence for a male-produced pheromone, but unlike *D. virilis*, it appeared to operate as a synergist of food odors, rather than an an attractant in its own right.

The counts of captured flies did not give information about the underlying behavioral mechanism—whether attraction had occurred or merely arrestment of randomly alighting individuals—but the upwind, hovering, casting approach to the vials, which was consistently observed, strongly supported attraction. Furthermore, since flies rarely departed after alighting at any food vial (with or without male extract), the increase in catch due to the extract reflected an increase in the number of flies approaching and alighting at those vials. The male extract was apparently perceived at a distance and contributed to the attractiveness of the vials.

Throughout this report, the number of flies caught per 3-min test represented a small fraction of the total flies in the olfactometer, frequently on the order of 1%. The percentage of bioassay flies responding depended to a large extent on the duration of pretest starvation. For example, when the flies were placed into the olfactometer 16 hr before testing, the mean catches were very large, being 115 for male extract + food versus 62 for food only ($N = 4$, 3 min/test). When the flies were stressed in this way, effects due to the male extract were less obvious (the ratio between treatments was less then 2:1), and most of the flies in the olfactometer were caught during the first few tests. When experimental conditions were adjusted to produce relatively small catches (i.e., tests begun just as the flies were beginning to move about), the flies discriminated between treatments more clearly, and furthermore, one group of bioassay flies served for many tests. For the purpose of identifying pheromone components, statistically clear differences between treatments were more meaningful than massive bioassay catches.

Although the fermented food was an effective coattractant for bioassays, it was difficult to maintain a consistent level of attractiveness from day to day. In addition, we felt that using a coattractant which was less active than fermented food might further clarify pheromonal effects and make pheromone identification more rapid. Solvents such as acetone or ethyl acetate (10 $\mu$l applied to the filter paper) were generally superior to food for bioassay purposes and were more convenient to use. For example (Table 1), the extract caused a threefold increase in the numbers responding to food but a 17-fold increase in the response to acetone. Although the absolute numbers responding to the extract-acetone mixture were lower than with food, the observed ratio to the acetone control

TABLE 1. BIOASSAY COMPARISONS AMONG MALE EXTRACT, FERMENTED FOOD, AND ACETONE

| Treatment | Mean bioassay catch[a] ($N$ = 20) | |
|---|---|---|
| Control | 0.1 c | |
| Male extract[b] | 0.9 c | |
| Acetone | 0.4 c | |
| Male extract + acetone | 6.8 b | (17 × increase over acetone) |
| Food | 7.5 b | |
| Male extract + food | 21.5 a | (2.9 × increase over food) |

[a] Means followed by the same letter were not significantly different in the log ($n$ + 1) scale at the 0.01 level (LSD).
[b] Male extract used at 1 fly equivalent/test.

was relatively large, and it was more consistent from day to day than with food. As noted above, the response toward the crude extract was poor when no coattractant was present. Acetone was used as the coattractant in the bioassay tests leading to the identification of the pheromone component. Compounds such as acetone and ethyl acetate are known to be produced by molds or yeasts (Fogleman, 1982) and could well influence the behavior of the flies under natural conditions. Such compounds have been previously shown to be attractants for *D. melanogaster* (Hutner et al., 1937; Rodrigues and Siddiqi, 1978).

*Purification and Identification.* After fractionation of the crude extract of males on silicic acid, only the 7.5% ether–hexane fraction, containing compounds with the polarity of esters, was significantly active (Table 2). By capil-

TABLE 2. BIOASSAY TESTS OF SILICIC ACID FRACTIONS FROM CRUDE EXTRACT OF MATURE MALES

| | Mean bioassay catch[b] ($N$ = 12) | |
|---|---|---|
| Name of fraction[a] | Fraction + acetone | Acetone |
| Hexane | 2.0 | 1.7 |
| 2.5% Ether–hexane | 3.9 | 2.8 |
| 7.5% Ether–hexane | 18.3*** | 3.6 |
| 25% Ether–hexane | 4.0 | 2.4 |
| Ether | 4.0 | 2.8 |
| Crude extract | 17.8*** | 3.1 |

[a] All fractions and extracts used at 5 male equivalents per test. Acetone was used at 10 $\mu$l per test.
[b] Differences from the control at the 0.001 level denoted by ***, no other differences were significant at the 0.05 level.

lary GLC, this fraction was predominantly (80%) one compound, which was suspected from previous work (Brieger and Butterworth, 1970) to be cVA. Comparison of mass spectra and GLC retentions of the fly-derived and synthetic (Sigma Chemical Co., St. Louis, Missouri) compounds and double-bond location by ozonolysis–GLC confirmed this identity.

*Bioassays with Synthetic Compounds.* The crude extract, 7.5% ether–hexane fraction, synthetic cVA (99+% pure), and fly-derived cVA (obtained from the 7.5% ether–hexane fraction by preparative GLC, 99+% pure) were bioassayed against each other (all at 1.5 μg cVA per test) and controls (Table 3). The synthetic cVA and fly-derived cVA were not significantly different ($P \cong 0.4$), but both surpassed the control ($P < 0.001$). However, the crude extract and 7.5% ether–hexane fractions did exceed the cVA samples in activity. Thus there was good evidence that cVA was a component of the aggregation pheromone but that one or more additional components also existed. The 7.5% ether–hexane fraction appeared to contain at least one component besides cVA. The additional components are under investigation. In bioassay, doses of synthetic cVA from 0.15 to 150 μg plus acetone were significantly more attractive than the acetone control (Table 4). Finally, synthetic cVA was bioassayed with food to confirm the activity of the ester with the more "natural" coattractant. Means for the cVA + food and food treatments were 3.4 and 0.5, respectively ($N = 36$, $P \ll 0.001$). Thus cVA operated similarly with both coattractants.

Both sexes responded similarly to cVA. For example, in Table 3, about 50% of the flies captured for each treatment were males. When only males or only females were placed in the olfactometer, significant responses were also seen. For males, mean catches ($N = 8$) for the cVA + acetone, acetone, and cVA alone were 2.1, 0.1, and 0.3, respectively, while for females, the means ($N = 8$) were 5.9, 1.1, and 0.3. In both cases, the combination differed from both other treatments at the 0.001 level.

TABLE 3. SOURCES OF cVA COMPARED IN BIOASSAY

| Source[a] | Mean bioassay catch[b] ($N = 40$) | Captured flies which were males (%) |
|---|---|---|
| Crude male extract + acetone | 9.7 a | 47 |
| 7.5% Ether–hexane fraction from males + acetone | 7.4 a | 50 |
| cVA from males + acetone | 4.7 b | 52 |
| Synthetic cVA + acetone | 4.0 b | 47 |
| Control (acetone) | 1.1 c | 60 |

[a] In each treatment there was 1.5 μg cVA per test.
[b] Means followed by the same letter were not significantly different at the 0.05 level.

TABLE 4. DOSE–RESPONSE TO SYNTHETIC cVA

| Treatment | Mean bioassay catch ($N$ = 24)[a] |
|---|---|
| 0.15 μg cVA + acetone | 3.0 b |
| 1.5 μg cVA + acetone | 4.2 b |
| 15 μg cVA + acetone | 8.6 a |
| 150 μg cVA + acetone | 8.6 a |
| Acetone (control) | 1.3 c |

[a]Means followed by the same letter do not differ significantly at the 0.01 level.

There appeared to be a considerable amount of structural specificity for cVA, as might be expected for a pheromone component. To test the importance of double-bond location, cVA + acetone was compared to (Z)-9-octadecenyl acetate (oleyl acetate) + acetone and to the acetone control. The means were 14.5, 5.6, and 4.1, respectively ($N$ = 24, 1.5 μg of ester per test). cVA differed from oleyl acetate at the 0.001 level, but oleyl acetate was not significantly different from the control. cVA was also compared to the free alcohol (Z)-11-octadecen-1-ol (cVOH). The means for cVA + acetone, cVOH + acetone, and the acetone control were 8.0, 1.4, and 2.4, respectively ($N$ = 12). cVA differed from cVOH and from the control at the 0.001 level, but the cVOH treatment and the control did not differ. Thus shifting the double bond to the 9 position or hydrolyzing the ester linkage destroyed the activity.

*Function and Emission of cVA.* The aggregation activity toward cVA was surprising because the chemical had been shown previously to be a mediator of close-range courtship behavior. It was found to be located in the male reproductive tract and was transferred to females during mating (Butterworth, 1969). cVA was shown to inhibit males from courting other males and also recently mated females (Jallon et al., 1981). This appears to be an example of pheromonal parsimony (Blum, 1977), in that one compound functions in more than one way, depending on ecological context.

More research is needed to fully understand the relationships between long-range attraction and close-range courtship in *D. melanogaster*, but a few comments can be made. First, cVA acting as a coattractant of food odors is not inconsistent with the habits of these flies, since mating, feeding, and oviposition usually occur at the same site. Any appropriate food source would be attractive to flies moving through the environment, but the flies would land preferentially at sites where cVA was being emitted, leading to aggregations, even when abundant food sources existed.

A question of primary importance is to determine under what circumstances cVA is emitted by the flies. To provide baseline information, the amounts of cVA actually present in the flies were determined. Only traces (0.02 μg/male)

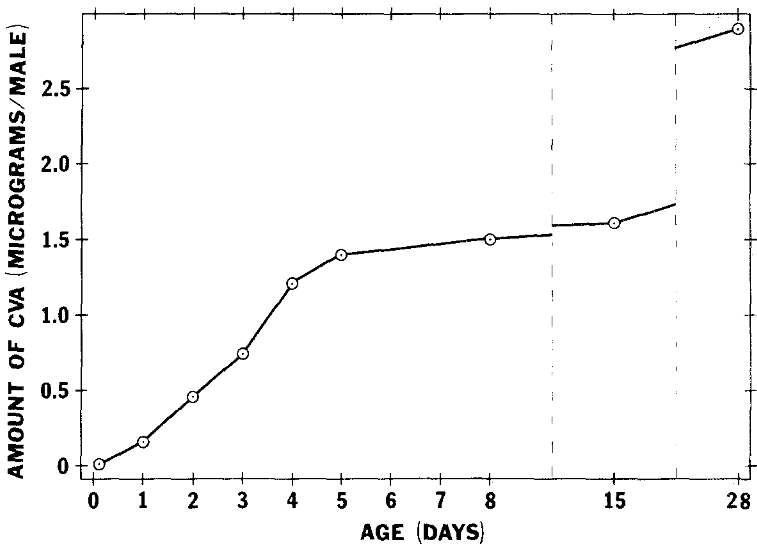


FIG. 1. Amounts of cVA extracted from virgin male *D. melanogaster* of various ages.

were found in 0- to 4-hr-old males (Figure 1), but amounts increased dramatically with age, being 1.4 μg/male at 5 days of age and as high as 2.9 μg/male at 4 weeks. The amounts measured in mature flies were higher than reported by Jallon et al. (1981). As reported previously (Butterworth, 1969), no cVA was found in virgin females.

Hexane rinses of rearing vials gave one measure of the amounts of cVA actually emitted by the flies. Although the ester was reported to be contained in the ejaculatory bulb (Butterworth, 1969), we found that low levels of cVA did accumulate in feeding vials occupied by virgin males (Table 5). At least 0.15

TABLE 5. AMOUNTS OF cVA EXTRACTED FROM REARING VIALS AFTER BEING OCCUPIED BY VIRGIN MALES FOR 2 DAYS (7 REPLICATIONS PER AGE)

| Age when placed into vials (days) | cVA recovered from vial (μg/male ± SD) |
|---|---|
| 0–1 | 0.05 ± 0.02 |
| 2–3 | 0.08 ± 0.03 |
| 4–5 | 0.08 ± 0.01 |
| 6–7 | 0.15 ± 0.04 |

μg/male were emitted by 6- to 7-day-old males, an amount which was only ca. 10% of that possessed by the flies, but an amount which was able to cause a significant increase in bioassay response (Table 4). If the extraction efficiency was similar to that for the standards, the true amount present may have been almost 0.5 μg/male. Thus, even a single mature male could reasonably make one food source more attractive than another, although the rates of cVA deposition and volatilization under field conditions remain to be studied. No cVA was detected in vials which had contained virgin females of any age.

The transfer of cVA from males to females reported earlier (Butterworth, 1969) seemed, at first, to be unrelated to aggregation. Yet, when females were placed in fresh rearing vials immediately upon completion of mating and the rearing vials extracted after 6 hr, a relatively large amount of cVA was recovered, 0.30 ± 0.08 (SD) μg/female, which was more than twice as great as the amount remaining in the females, 0.13 ± 0.03 μg/female. Thus any site at which successful mating took place might become even more attractive to other flies. It is unknown whether the cVA was deposited during oviposition, although numerous eggs were found in the rearing vials. It was noted previously that the amount of cVA extracted from mated females decreased with time after mating (Jallon et al., 1981), and Mane et al. (1983) reported that the cVA could be hydrolyzed to the free alcohol, cVOH, by an esterase system. However, in our experiment, relatively massive amounts of cVA were expelled from females without being hydrolyzed.

It seemed contradictory that male bioassay flies, which were old enough to possess cVA, nevertheless responded to cVA in bioassay. One possibility was that cVA disappeared during the prebioassay starvation period. However, the amounts of cVA present in males starved in the olfactometer for 6 hr before extraction and in parallel groups of males which were not starved were similar [means were 0.80 ± 0.02 (SE) μg/male and 0.82 ± 0.02 μg/male, respectively]. Thus it is probable that the males did not emit their cVA during tests, even though they possessed it. In any event, the response toward test vials remained fairly consistent during 3-min tests—males landing at the test vial did not accelerate the subsequent rate of response.

In summary, extracts of mature males were shown to be significant synergists of food odors in attracting both males and females of *D. melanogaster*. The major active component in these extracts was determined to be cVA. Although an elegant body of evidence exists concerning the function of cVA in close-range courtship behavior—that it discourages males from courting other males and recently mated females—the possibility of additional functions for this compound must not be overlooked. The emission of cVA at low levels by virgin males and at higher levels by recently mated females, together with the consistent bioassay responses observed toward cVA in the olfactometer, demonstrate that a broader view of the role of cVA must be taken.

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# GASTER FLAGGING BY FIRE ANTS (*Solenopsis* spp.): FUNCTIONAL SIGNIFICANCE OF VENOM DISPERSAL BEHAVIOR

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**Abstract**—Behavioral and chemical studies with laboratory colonies indicate that the imported fire ant *Solenopsis invicta* Buren (Myrmicinae) disperses venom through the air by raising and vibrating its gaster (i.e., "gaster flagging"). This mechanism of airborne venom dispersal is unreported for any ant species. Foraging workers utilize this air-dispersed venom (up to 500 ng) to repel heterospecifics encountered in the foraging arena, while brood tenders dispense smaller quantities (~1 ng) to the brood surface, presumably as an antibiotic. Brood tenders removed from the brood cell and tested in heteropspecific encounters in the foraging arena exhibited the complete repertoire of agonistic gaster flagging behavior. These observations suggest that airborne venom dispersal by workers is context specific rather than temporal caste specific and that workers can control the quantity of venom released.

**Key Words**—Ants, *Solenopsis invicta*, Hymenoptera, Formicidae, gaster flagging, alkaloids, defensive behavior, venom, antibiotic, caste.

## INTRODUCTION

Functional parsimony is a recurring theme in the evolution of ant venoms, sting morphology, and related behaviors. Whereas social bees and wasps employ their stings almost exclusively for defense (but see Maschwitz, 1964; Post and Jeanne, 1983), ants use their stings for defense, prey capture, and pheromone dispersal (Wilson, 1971; Bradshaw and Howse, 1984). Here, we present a remarkable venom dispersal mechanism and behavioral correlates of venom alkaloid activity in the fire ant *Solenopsis invicta* Buren (Myrmicinae).

*S. invicta* is a South American ant that has attained introduced pest status in the southearstern United States (Lofgren et al., 1975). It is best known for its aggressive behavior and its painful sting. *S. invicta* venom is primarily (>95%) composed of piperidine alkaloids (2-alkyl or alkenyl 6-methyl piperidines). The in vitro insecticidal and antibiotic properties of the piperidine alkaloids have been established in laboratory assays (Blum et al., 1958; Jouvenaz et al., 1972). We have investigated the adaptive significance of these venom characteristics, particularly with respect to "gaster flagging" behavior (Adams and Traniello, 1981) in which workers raise and vibrate the gaster, frequently while extruding the sting. This behavior was noted during laboratory and field observations of *S. invicta* colonies by us and others (Bhatkar et al., 1972). We hypothesized that during gaster flagging, *S. invicta* workers dispersed venom through the air.

We chose to address two contexts during which gaster flagging is observed. *S. invicta* workers undergo age polyethism whereby young workers tend brood, while older workers do most of the foraging (Mirenda and Vinson, 1981; cf. Wilson, 1978). Gaster flagging was observed among workers tending brood as well as among foraging workers confronting heterospecifics in the foraging arena. It occurred to us that venom directed at heterospecifics encountered in the foraging arena could function as repellent, while venom directed to the brood or the surrounding brood chamber could reduce the likelihood of microbial infection.

In the following studies, we (1) more fully describe gaster flagging by *S. invicta*, (2) show that venom is dispersed through the air by gaster flagging during heterospecific confrontations, and (3) demonstrate that worker-derived venom is present on the surface of the brood.

## METHODS AND MATERIALS

*Analysis of Gaster Flagging Behavior.* Ants tested were members of single queen colonies collected four to six months previously and maintained at the ARS, USDA Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida. Colonies contained more than 10,000 individuals and included immatures at all developmental stages. Colonies were maintained in plastic Petri dish cells (diameter = 14.0 cm) with Castone® floors at 26–27°C on a diet of honey-water, fly pupae, and hard-boiled egg. These brood chambers were placed in plastic trays (52.0 × 39.0 × 7.5 cm) that served as foraging arenas (Banks et al., 1981). The sides of the tray were Fluon® coated.

The behavior of brood tenders in the brood chamber was observed in three different *S. invicta* colonies under an adjustable three diopter illuminated magnifier during a total of 4.3 h. Confrontation behavior was observed following introduction of individual *S. invicta* foragers or brood tenders into (1) the foraging arena of heterospecific (*S. geminata*) colonies (*N* = 11 trials) or (2) Petri

dish arenas (diameter = 5.4 cm) housing 8–12 *S. geminata* workers (*N* = 13 trials). Data were collected for 10 min post-introduction. Interspecific interactions in the Petri dish arenas were observed under a dissecting microscope fitted with an ocular micrometer. These interactions were also filmed with a Fujica ZC1000 Single-8 camera fitted with a Tamron Tele-Macro lens (1 : 1).

*Airborne Venom Dispersal by Foragers.* Two experiments were performed. In the first, encounters in Petri dish arenas were staged between groups of 8–12 *S. invicta* and *S. geminata* workers. When alarmed individuals began gaster flagging, a TLC plate impregnated with iodoplatinate reagent (Touchstone and Dobbins, 1978) was held 1–2 cm from the tip of the sting while the ant was prodded from the rear and side with forceps. (Iodoplatinate turns from red to either white or blue in the presence of alkaloids.) An impregnated plate was discarded whenever it was potentially contaminated due to contact with an ant, the arena surface, or forceps that had contacted either. The size and pattern of venom droplets on the TLC plate were analyzed under the dissecting microscope. Tests with alkaloid standards derived from extirpated *S. invicta* poison sacs indicated that as little as 5 ng/spot (minimum spot diameter = 0.02 mm) could be visualized by this method.

In the second experiment, lone *S. invicta* foragers were introduced into Petri dishes housing 10–12 *S. geminata* workers. During confrontations, the *S. invicta* intruder would invariably gaster flag at resident *S. geminata. S. geminata* workers making their initial approach to the introduced *S. invicta* worker were occasionally repelled without contacting the intruder's sting. Eight of these *S. geminata* were immediately collected and swirled in hexane (250 $\mu$l) for 30 sec. An internal standard (eicosane, Applied Science) was added and the sample analyzed for species-specific *S. invicta* piperidine alkaloids (Brand and Blum, 1973) by gas–liquid chromatography: DB-1 fused silica capillary column (J. and W. Scientific, Inc.), 15 m × 0.33 mm, splitless; Varian 3700, FID, temperature program = 150–200 at 2°C/min; DB-225 fused silica capillary column (J. and W. Scientific, Inc.), 30 m × 0.26 mm, splitless; Varian 3700, FID, 200°C isothermal.

*Worker-Derived Venom on the Brood.* A brood cell containing workers and brood was frozen and then the brood separated from brood tenders in an air-stream (Stringer et al., 1972). The brood sample (*N* = 8000 immatures) was examined to ensure that workers were absent and then rinsed for 1 min in 2 : 1 chloroform–methanol (5 ml). This rinse was divided into two equal subsamples, each of which was reduced to 25 $\mu$l under a stream of nitrogen and then brought to 500 $\mu$l with hexane. Venom alkaloids were isolated and purified by (1) washing three times with 0.5 N sulfuric acid (200 $\mu$l) (2) separation of the aqueous phase followed by basification wth 1.5 N potassium hydroxide (250 $\mu$l), and (3) extraction of the alkaloids from the basified aqueous phase three times with hexane (200 $\mu$l). Internal standard was added to the hexane extract prior to GLC analysis (as above).

In order to assess potential contamination from endogenous larval and pupal alkaloids, we quantified the alkaloid contents of five extirpated pupal poison glands, and then determined the percent alkaloids extractable from whole workers by our rinse method. In addition, we quantified potential contamination from worker venom extruded in the airstream sorting device. We lined the apparatus used to separate brood from brood tenders with filter paper and conducted another brood/worker separation. The filter paper was then cut into strips, extracted with hexane, and vacuum-filtered. The filtrate was concentrated under nitrogen and analyzed by GLC for the presence of venom alkaloids.

## RESULTS

*Gaster Flagging Behavior.* We recognized three distinct types of gaster flagging in *S. invicta*, hereafter referred to as the "headstand," "defensive flag," and "brood flag." The former two were exhibited only by workers during interspecific encounters.

Both brood tenders and foragers exhibited the headstand and/or defensive flag during the arena encounters. While performing the headstand (Figure 1), workers straighten the rear legs, elevate the petiole approximately 70–90° to the substrate, and extrude the sting. They then vibrate the gaster in the anteroposterior plane. A drop of venom up to 0.2 mm in diameter appears at the tip of the sting within several seconds. The "headstand" appears to be a nonoriented, general response to initial contact with a heterospecific or in response to grappling or other agonistic interaction between nestmate(s) and heterospecifics occurring within 2.0–3.0 cm. Individual *Solenopsis* of both species often gaster flagged in the headstand position near a grappling pair.

Defensive flagging is distinguished from the headstand by four criteria: (1) During defensive flagging, the gaster is clearly oriented toward specific intruders (Figure 2). (2) The gaster is elevated no more than 45°. (3) Although the sting is extruded, no distinct venom droplet is observed. (However, when defensive flagging follows or is interrupted by headstanding, venom may be present at the sting tip.) (4) Gaster vibration during defensive flagging is much more vigorous (i.e., the amplitude of gaster deflection is greater), and lateral as well as anteroposterior gaster deflection can be observed.

During our observations of brood chambers, we noted 161 incidents in which brood tenders raised and either vibrated or repeatedly shook their gasters while contacting or standing within 1.0 cm of brood. When they gaster flagged in the brood chamber, brood tenders elevated the gaster no more than 45°. However, the intensity of gaster vibration was highly variable. In nine instances, ants extruded their stings, but we observed no venom droplets.

*Airborne Venom Dispersal by Foragers.* Ants responded to prodding by either continuing to headstand or by flagging extruded venom at the TLC plate.

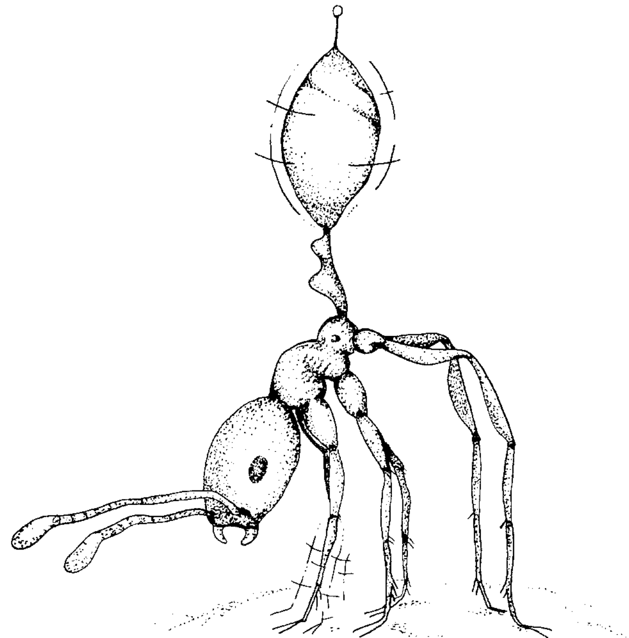

FIG. 1 The headstand or initial, nondirectional gaster flagging posture assumed during heterospecific encounters. Stereotyped elements include 90° gaster elevation, vertical gaster vibration, sting extrusion, venom droplet secretion, lowered head, open mandibles, and foretarsal tapping.

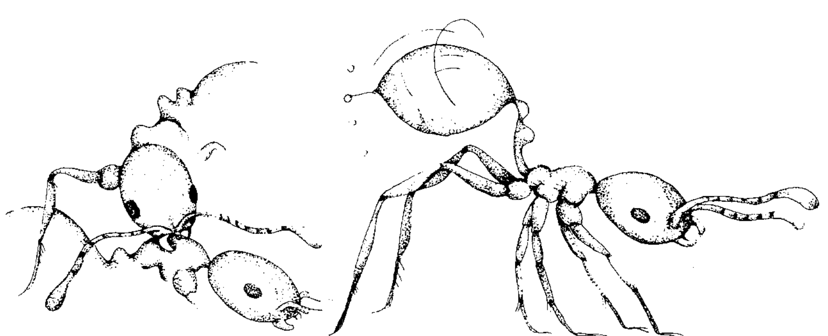

FIG. 2. The defensive flag, here directed at an immobilized heterospecific. This behavior is further distinguishable from the headstand in that the gaster is elevated much less than 90°, the venom droplet may or may not be present, and the gaster is vigorously shaken in both vertical and horizontal planes.

Plates at whch ants did not direct "defensive flagging" ($N = 6$) contained no visible evidence of venom alkaloids. Six of eight plates at which ants had directed defensive flagging did, however, contain venom alkaloids. Analysis of these plates revealed 4–11 venom droplets/mm$^2$ in the exposed areas. The droplets were assigned to two discrete size classes: (1) diameter = 0.08–0.13 mm, and (2) diameter = 0.02–0.03 mm. The larger droplets constituted from 9.9% (1 of 11) to 100% (4 of 4) of all venom droplets noted in a sampling area. No ordered pattern of droplets was apparent.

In our second experiment, *S. geminata* workers were occasionally repelled by gaster flagging *S. invicta* over a distance of up to 1.5 cm. Behaviors of repelled ants included rapid withdrawal, antenna dragging, and grooming. In addition, species-specific *S. invicta* venom alkaloids were recovered from the cuticle of *S. geminata* workers visibly repelled by gaster flagging (Figure 3A–C). Total venom present in the pooled sample of eight ants was between 3.5 and 4.0 μg.

*Worker Derived Venom on the Brood.* The two brood rinse subsamples yielded 1.13 ng alkaloids/immature and 0.92 ng alkaloids/immature (Figure 4). When *S. invicta* adults were subjected to the same rinse technique, they yielded less than 0.002% of the total available alkaloids from the surface and poison sacs. We assumed that all 4000 immatures per subsample contained the amount of alkaloids measured in the extirpated pupal posion sac (= 257 ng/pupa) and that our technique extracted 0.002% of these alkaloids (approximately 5 pg/immature). This figure represents 0.48% and 0.55% of the total alkaloid content in our two rinse samples. Venom alkaloids quantified in two samples of filter paper lining the ant sorting device were present at 13.0 and 16.3 pg/immature. Thus, between 1.25 and 1.48% of the venom in our brood rinses was potentially a result of contamination from worker venom extruded during the sorting process. Total venom on the brood surface excluding the maximum possible contamination from poison glands of workers and immatures was calculated to be 1.04 and 0.80 ng/immature, respectively.

## DISCUSSION

It has been stated (Schmidt, 1978) that plasticity of venom composition and venom dispersal mechanisms in ants has been an important factor in ant domination of terrestrial habitats. Other species of myrmicine ants secrete venom droplet(s) to the sting tip. Volatile components of extruded venom are known to function in worker recruitment (i.e., "tandem calling") (Möglich et al., 1974; Möglich, 1979) and sexual calling (Buschinger, 1968, 1971). In addition, other species wipe venom droplets on antagonists as part of either raiding (Hölldobler, 1973; Blum et al., 1980) or interference competition strategies (Adams and

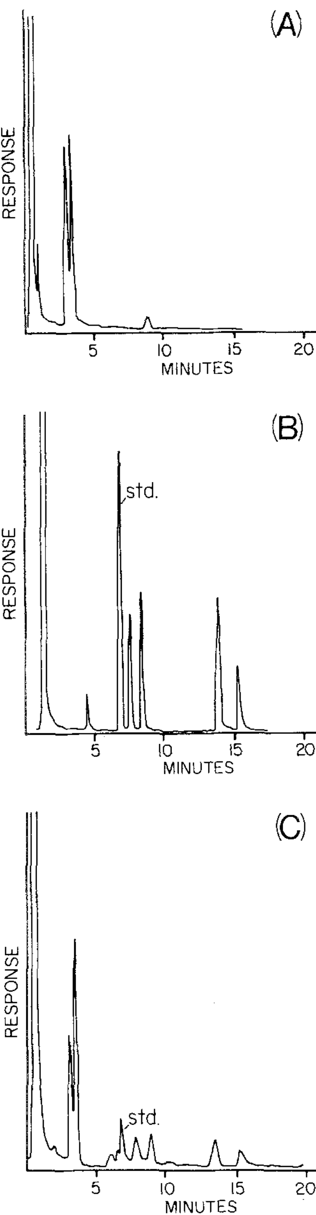


FIG. 3. Gas chromatogram demonstrating the presence of air-dispersed *S. invicta* venom alkaloids on *S. geminata* cuticle. (A) *S. geminata* cuticle rinse. (B) *S. invicta* venom alkaloids from extirpated worker poison sacs. (C) *S. geminata* cuticle rinse following arena encounter with gaster flagging *S. invicta*. Std = internal standard.

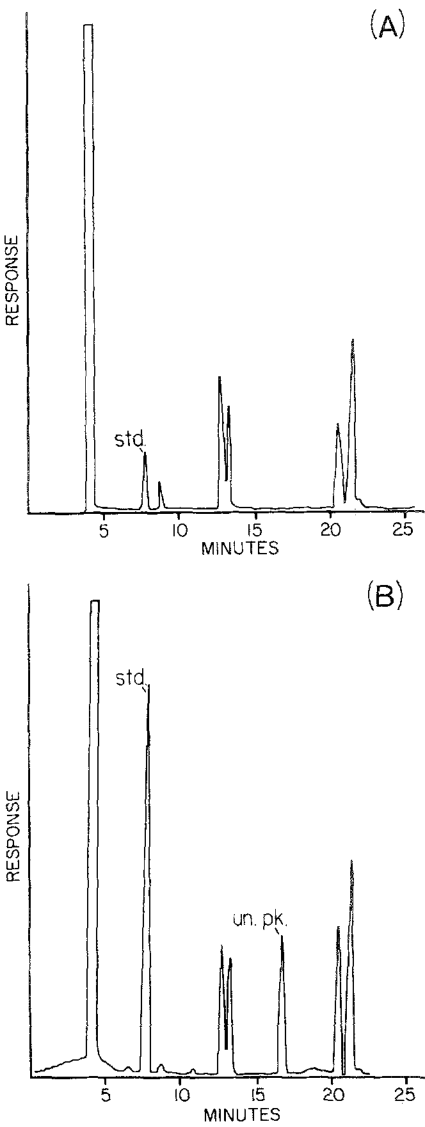


FIG. 4. Gas chromatogram demonstrating the presence of worker-derived venom alkaloids on the surface of *S. invicta* brood. (A) *S. invicta* venom alkaloids from extirpated worker poison sacs. (B) *S. invicta* brood rinse. Std = internal standard, un. pk. = unidentified peak.

Traniello, 1981; Hölldobler, 1982). Data presented here offer what is to our knowledge the first evidence of airborne dispersal of venom droplets by the sting apparatus of an ant, although we suggest that it may be a general phenomenon in this subfamily. However, a "spray sting type" of mechanism (Maschwitz and Kloft, 1971) has been previously observed in several species of vespid wasps (reviewed in Hermann and Blum, 1981).

The biomechanics of airborne venom dispersal by gaster flagging may have a precedent in man-made physical analogs. Rayleigh (1879) first proposed that directed streams of small droplets could be generated by a mechanically vibrated jet of liquid. More recently, Mason et al. (1963) produced controllable, reproducible streams of 30- to 500-$\mu$m droplets with a hypodermic needle (bore diameter = 100 $\mu$m) vibrated at resonant frequencies with an amplitude of several millimeters. We suggest that *S. invicta* (and congenerics) utilize a similar principle to produce a venom aerosol.

Venom dispersed by gaster-flagging *S. invicta* visibly repels heterospecifics and presumably functions as an antiseptic in the brood chamber. Our analysis of airborne venom dispersal by foraging workers suggests that during the "headstand" behavior workers pump large quantities of venom from the poison reservoir into the sting. Although not detected in this study, volatile components with potential pheromonal function may be released from the secreted droplet. Data indicate that as much as 500 ng of venom may be dispersed when directed at heterospecifics during defensive flagging. This figure represents 2.5% of the total alkaloid content of a typical *S. invicta* worker (Vander Meer, unpublished data).

In response to alarm, invasion, or other disturbance, brood tending *Solenopsis* workers typically pick up brood and flee the brood chamber. However, brood tenders removed from the brood chamber and introduced into arenas containing heterospecifics exhibited the full repertoire of confrontation gaster flagging behavior observed in foragers (i.e., the headstand and defensive flag). Although the product of artificial laboratory manipulation, this observation suggests that mechanisms of chemical defense in *Solenopsis* are not specific to a particular *S. invicta* temporal caste but, rather, are context specific.

With respect to venom on the brood surface, our data do not exclude the possibility that venom is transferred to the brood as a consequence of worker/brood contact (e.g., by grooming). However, if gaster flagging is the method of venom application, the small quantity of venom present ($\sim$1 ng, presumably evenly distributed on the brood surface) argues in favor of an aerosol with droplet diameters much less than 20 $\mu$m. Recall that our method of visualizing *Solenopsis* venom alkaloids permitted detection of no less than 5.0 ng venom/spot—a spot 20–30 $\mu$m in diameter. Based on physical models (Mason et al., 1963), it can be hypothesized that venom flow rate is the essential determinant of droplet diameter, although sting orifice diameter, vibrational frequency, and

amplitude may also play a role. Future efforts should include quantification and comparison of these features of gaster flagging mechanics. In this manner, a more complete understanding of how *S. invicta* workers regulate the amount of potentially lethal alkaloids that they apply to the brood surface may be achieved.

Selection pressures contributing to the evolution and maintenance of insecticidal repellents and antibiotic secretions in ants include (1) predation and/or competition due to other arthropods, especially ants and (2) conditions favoring bacterial and fungal growth. Although such pressures may vary in intensity, they should be ubiquitous among most soil-inhabiting ants species. Other myrmicine ants rely on repellents produced in the poison gland (Hermann and Blum, 1981), and antibacterial secretions derived from the metapleural gland (Maschwitz et al., 1970; Wilson 1971). Our data and those of Blum et al. (1958) indicate that the production and disperal of antibiotic exocrine materials in *Solenopsis* spp. is a function of the poison gland and sting apparatus. Furthermore, our laboratory (Alvarez, personal communication) has documented the absence of antibiotic activity in the metapleural gland of *S. invicta*. These findings argue in favor of an alternative role for the metapleural gland in fire ant ecology. Evidence in support of this hypothesis was recently presented by Jaffe and Puche (1984), who reported the production of colony-specific territorial pheromones in the metapleural glands of *S. geminata*. Antibiotic metapleural gland secretions of other Myrmicines tested by Maschwitz et al. (1970) did not function in either territoriality or nestmate recognition.

Lastly, we suggest that the internal sequestration and facultative dispersal of antimicrobial venom alkaloids by *S. invicta* may have implications for current theories of ant–plant interactions. Based on metapleural gland secretions of four geographically and ecologically different ant species (Schildknecht and Koob 1970, 1971), Beattie et al. (1984) suggested that antimicrobial secretions of ants interfere with pollen function (Iwanami and Iwadare 1978) and plant seed set. Since metapleural gland secretions are distributed over the entire surface of the ant body (Maschwitz et al. 1970), Beattie et al. (1984) hypothesized that antibiotic ant secretions have constrained the evolution of ant pollination. Quantification of venom alkaloids present on the body surface of *S. invicta* foragers in conjunction with knowledge of the effect of the venom alkaloids on pollen may provide one test of this hypothesis.

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